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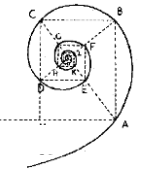

DOTTORATO IN MEDICINA MOLECOLARE

E TRASLAZIONALE

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TESI DI DOTTORATO DI RICERCA

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PEPTIDE-CONJUGATED MORPHOLINO OLIGOMERS FOR TREATMENT OF SPINAL MUSCULAR ATROPHY

Dottoranda: **Agnese RAMIREZ**

Matricola N°***R10958-R21***

Tutor: Prof. Giacomo COMI

Co-Tutor: Prof.ssa Stefania CORTI

Coordinatore del dottorato: Prof. Riccardo GHIDONI

Sommario

L'Atrofia Muscolare Spinale (SMA) è una patologia neuromuscolare autosomica recessiva che colpisce i motoneuroni, ed è, ad oggi, la prima causa genetica di mortalità infantile. E' causata da mutazioni in omozigosi del gene Survival Motor Neuron 1 (SMN1), che danno luogo alla produzione di insufficienti livelli della proteina SMN.

Da un punto di vista patologico, la SMA è caratterizzata da una perdita progressiva dei motoneuroni (MN) nelle corna anteriori del midollo spinale a cui consegue una perdita delle funzioni muscolari, paralisi e morte prematura.

Da quest' anno è stato approvato il primo farmaco per la SMA, Nusinersen, un oligonucleotide antisenso (ASO). Infatti, una delle possibili strategie che permettono di aumentare i livelli di proteina SMN funzionante è l'uso di ASO in grado di modulare lo splicing dell'mRNA di SMN2, il gene paralogo di SMN1.

Attualmente sono utilizzati in trial clinici ASO con diverse strutture chimiche, inclusi gli ASO con una chimica di tipo Morfolino (MO) studiati in questo lavoro. Gli oligomeri di tipo Morfolino sono particolarmente utili per applicazioni in vivo grazie al loro buon profilo di sicurezza ed efficacia.

Lo scopo di questo studio è di migliorare l'efficacia del MO e di contribuire a risolvere il problema della sua relativa bassa biodisponibilità tissutale. Infatti gli ASO in generale non riescono a superare la barriera emato-encefalica e devono essere somministrati attraverso un'iniezione relativamente invasiva intratecale nel liquido cefalorachidiano per raggiungere il sistema nervoso centrale.

In primo luogo, per migliorare l'efficacia degli oligomeri di tipo MO, abbiamo testato nuove sequenze di MO che si appaiano alla regione identificata come intronic splicing silencer N1 (ISS-N1) di SMN2 sia in vitro che in un modello murino transgenico di SMA (SMAΔ7).

L'uptake cellulare e tissutale e i profili farmacologici degli ASO possono essere migliorati in modo significativo attraverso la loro coniugazione con cell-penetrating peptides (CPP), ovvero peptidi che riescono a penetrare nelle membrane cellulari, ma questo approccio non è ancora stato utilizzato nella SMA. Quindi, come seconda fase dello studio, ci siamo proposti di valutare un

approccio terapeutico per la SMA utilizzando sequenze MO coniugate a diversi tipi di CPP. Abbiamo testato tre diversi CPP, Tat, R6 e (RXRRBR)2XB che sono stati associati alla sequenza di MO [HSMN2Ex7D(-10,-34)] da noi già precedentemente validata.

Abbiamo somministrato i coniugati ottenuti in modelli murini di SMA ad uno stadio pre-sintomatico e sintomatico della malattia per determinare il loro effetto terapeutico rispetto al MO non coniugato.

Abbiamo dimostrato che l'ottimizzazione della sequenza target può migliorare l'effetto della correzione dello splicing attuata da MO e che l'effetto della co-somministrazione di più di una sequenza di MO è maggiore rispetto alla somministrazione di una singola sequenza. Inoltre abbiamo dimostrato che i coniugati CPP-MO sono in grado di aumentare sia i livelli di proteina SMN nel sistema nervoso centrale sia la sopravvivenza media e le performance motorie nel modello murino SMA dopo una somministrazione intracerebroventricolare (ICV) e intravenosa (IV).

I nostri risultati hanno prodotto dei dati preliminari in grado di sostenere che la coniugazione degli oligomeri di tipo MO ai CPP può essere una strategia attuabile per aumentare l'uptake cellulare dopo una somministrazione intratecale e per veicolare il MO nel sistema nervoso centrale dopo una somministrazione sistemica non invasiva. I risultati ottenuti saranno utilizzati per la selezione del CPP più efficiente che sarà studiato ulteriormente con analisi più approfondite e sarà un punto di partenza per lo sviluppo di futuri trial clinici.

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease and the first known genetic cause of infant mortality. It is caused by the homozygous mutations in the Survival Motor Neuron 1 (SMN1) gene, resulting in deficiency of functional SMN protein.

The pathologic aspect of SMA is the progressive loss of Motor Neurons (MNs) in the ventral horn of the spinal cord, which causes the loss of muscle function, paralysis and premature death.

A first drug for SMA, Nusinersen an antisense oligonucleotide (ASO) has been recently been approved. In fact, one of the most promising strategies in order to increase the SMN protein levels is the use of ASOs to redirect the splicing of the paralogous gene SMN2 to increase the production of the functional SMN protein.

ASOs with different chemical structure are currently used in clinical trials, included the ASO with Morpholino (MO) chemistry studied in this work. MO oligomers are particularly suitable for in vivo applications thanks to their optimal safety and efficacy profile.

One of the issues related to the administration of ASOs is that they are not able to cross the blood-brain barrier and they must be administered by relatively invasive intrathecal injection to reach the central nervous system (CNS). Therefore, we aimed to improve the efficacy of MO and to develop a non-invasive systemic delivery in an in vivo SMA murine model.

First, to increase the efficacy of MO oligomers, we tested, both in vitro and in vivo, four new MO sequences targeting the region involved in the alternative splicing of SMN2 mRNA, called intronic splicing silencer N1 (ISS-N1).

Simultaneously, to improve the delivery of MO to affected tissues, we conjugated it to cell-penetrating peptides (CPPs), an approach already used to enhance the cellular and tissue uptake and the pharmacological profile of ASOs, but never explored in SMA.

We tested three different CPPs: Tat, R6, and (RXRRBR)2XB, which were linked to our already validated MO sequence [HSMN2Ex7D(-10,-34)]. We

administered the conjugates in pre-symptomatic and symptomatic SMA mice to determine their therapeutic efficacy versus unconjugated-MO.

We showed that optimization of the target sequence can enhance the splice-correction action of MO oligomers and that the effect of the co-administration of different MO sequences is superior to the delivery of a single sequence. Furthermore, we showed that CPP-MO compounds can up-regulate the SMN protein into the CNS of SMAΔ7 transgenic murine models as well as their average lifespan and motor performance after local and systemic injection.

Our data proved that the CPP-MO conjugates can be a suitable strategy to increase the cellular uptake after an intrathecal administration and to deliver MO oligomers to the CNS after a systemic injection. The presented results provided the basis for the selection of the most efficient CPPs and will set the stage for future clinical trials.

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LIST OF ABBREVIATIONS

SMA: Spinal Muscular Atrophy
SMN: Survival Motor Neuron
snRBPs: small nuclear riboproteins
SC: subcutaneous
IP: intraperitoneal
IV: intracerebroventricular
NMJ: neuromuscular junctions
AVV: adeno-associated viral vector
iPSCs: induced pluripotent stem cells
iPSCs-WT: wild-type iPSCs
iPSCs-SMA: SMA- induced pluripotent stem cells
ESCs: Embryonic stem cells
OCT4: octamer-binding transcription factor 4
SOX2: sex determining region Y - box 2
NANOG: homebox protein nanog
Klf4: krueppel-like factor 4
MN: motor neuron
ESSs: Exonic Splicing Silencers
ISSs: Intronic Splicing Silencers
ESEs:Exonic Splicing Enhancer
SR proteins: proteins rich in serine-arginine
hnRNPs: heterogeneous nuclear ribonucleoprotein
ASF: alternative splicing factor
SF2: Splicing Factor 2
U2 snRNP: U2 class of small nuclear ribonuclear protein
U2AF: U2 auxiliar factor
siRNA: small interfering RNA
ISS-N1: intronic splicing silencer N1
Gems: Gemini of coiled bodies
PCR-RFLP: Restriction Fragment Length Polymorphism
LV: lentiviral vectors
BBB: blood brain barrier
HDAC: histone deacetylase enzyme
ASOs: Antisense oligonucleotides
DMD: Duchenne Muscular Dystrophy
PEG: polyethylene glycol
NPs: nanoparticles
CPP: cell-penetrating peptide
SeV: non-integrating Sendai virus
FBS: fetal bovine serum

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Figure 22: Brain samples of SMA mice treated with the different CPP-MO conjugates, unconjugated MO or scr-MO by intrathecal injection.

Figure 23: Brain samples of SMA mice treated with the different CPP-MO conjugates, unconjugated MO or scr-MO by intravenous injection.

Figure 24: none of the untreated mice (left) can perform the righting reflex, thus to reposition all four paws on the ground when placed onto their back, after postnatal day 6 (P6) while R6-MO treated mice were able to perform the righting reflex even after P6.

Figure 25: MO treated mice was able to complete the rotarod test and presented an overall aspect similar to an healthy wild-type mice.

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1. INTRODUCTION

1.1 SPINAL MUSCULAR ATROPHIES

Spinal muscular atrophies (SMAs) encompass a subset of hereditary autosomal recessive neuromuscular diseases characterized by an early onset, usually in childhood, and a selective and progressive death of lower motor neurons in the brainstem and spinal cord. As a consequence of this loss, patients affected by this disease show a progressive proximal muscle weakness, atrophy and paralysis, which are usually symmetrical.

The heterogeneity of these atrophies are due to the variability of clinical features (age of onset, severity of the disease and pattern of muscular involvement) and of genetic features (causative gene defect and transmission mode).

The most common form, which belongs to Proximal Muscular Atrophy with childhood onset, is an autosomal recessive disease (SMA5q, OMIM #253300) with an incidence of 1 in 6000 to 1 in 10000 live births, with a carrier frequency of 1/40-1/60 60 [1, 2]. The genetic cause was found in a mutation or homozygous deletion in the telomeric copy (*SMN*1) of *SMN* (Survival Motor Neuron) gene, on 5q chromosome, which causes a depletion of SMN protein[3].

In the following study we will analyze the SMA5q. The typical clinical aspects present in SMA5q are generalized muscle weakness and atrophy. The pathology is characterized by a progressive and symmetrical muscle involvement, weakness and atrophy resulting in the loss of voluntary control of limbs and trunk movements, postural, swallowing and breathing. The intellectual functions are conserved and there is no involvement of sensory neurons.

From the analysis of patients' tissues, there is an evident sign of gliosis in the absence of demyelination by the anterior horn of the spinal cord and reduction in motor neurons number. Surviving motor neurons show both signs of degeneration, indicating an ongoing process of neuronal death, and signs of immaturity: they have a small size, are pyknotic, and have the Nissl substance poorly developed. Axons analysis at the level of the ventral roots show axons surrounded by a single Schwann cell, a lower density and a high percentage of poorly myelinated axons, while in the neuromuscular junctions neurofilaments aggregates are detected at the presynaptic level. The motor plates are smaller and structurally underdeveloped. Muscle biopsy also shows groups of hypertrophic fibers in a more general atrophic context.

1.1.1 HISTORY OF THE DISEASE

In 1891, Werdnig and Hoffman described SMA, asserting that the key pathological features of the disease were the atrophy of skeletal muscles and degeneration of lower motor neurons (MNs) in the anterior horns of spinal cord. Lately, respectively in 1899 and 1903, Sylvestre and Beevor described the severe form of SMA[4]. In the 1950s, milder form of SMA have been described: they were characterized by conserved ability to walk until adult life and prolonged lifespan.

In 1995, Lefebvre and colleagues discovered the disease-causing gene of SMA [3], making the diagnosis of the pathology possible through a simple molecular blood test, leading to the enhancement of early diagnosis and the establishment of standards of care for patients [5, 6].

The causative gene of SMA disorder was found in the survival motor neuron (*SMN*) gene [3] on chromosome 5q13 [7]. After a finest genetic characterization of the disease, it was described that the human genome harbors two forms of *SMN* gene encoding for the same

protein: a telomeric form (the *SMN*1 gene) and a centromeric form (*SMN*2 gene). The coding sequence of *SMN*2 gene differs from the one of *SMN*1 gene for few nucleotides and in particular for a C to T substitution in position 840. This change is essential for the transcriptional step. In fact, the transcription of *SMN*1 gene produces a full-length mRNA, which is translated in a full-length, functional SMN protein, while the C to T transition in *SMN*2 leads to the exclusion of exon 7 from the majority of SMN2 transcripts, causing the production of a truncate form of SMN2 protein, which is not functional and rapidly degraded into the cytoplasm. Only a small percentage (10%-15%) of *SMN2* transcript incorporates the exon 7 and it is translated in a functional full-length SMN protein.

Further analysis showed that the number of copies of *SMN*2 is inversely proportional to the severity of the disease [8]. Even if the SMA phenotype is primary related to the number of *SMN*2 copies, there are also other genetic variations that can modify and modulate it. There is, for example, a G to C substitution at the 859 position in the *SMN*2 gene which increases the number of *SMN*2 transcripts that retain exon 7. The increase of full-length SMN protein is associated with milder manifestations of the disease, even in the presence of only two copies of *SMN*2 [9].

1.1.2 CLINICAL CLASSIFICATION

SMA 5q is clinically classified into four groups, according to the age of onset and the highest motor function achieved by affected subjects (**Table 1**). A fifth group (type 0) has been added to describe as a severe form characterized by pre-natal onset and death within 3 weeks after birth[10].

SMA TYPE	AGE OF ONSET	HIGHEST MOTOR FUNCTION ACHIEVED	LIFE EXPECTANCY
Type 0	Pre-natal	None	Less than 1 month
Type I	0-6 months	Never sit unassisted	Less than 2 years
Type II	7-18 months	Can sit unassisted	Between 2 years and 4 years
Type III	After 18 months	Can stand and walk unaided	Normal life expectancy
Type IV	Second or third decade	Can stand and walk unaided	Normal life expectancy

Table 1: Clinical classification of SMA

This classification has several clinical advantages, even though SMA patients do not have all the characteristics of a specific type, so prognostic information cannot be constantly extrapolated.

• **Type I** (OMIM 253300): named Werdnig- Hoffman disease, is the most common and severe form. Infants never gain the ability to sit unsupported. The head control is usually missing.

• **Type II** (OMIM 253550): named Dubowitz Disease, is the intermediate chronic infantile onset form. Affected children reach the ability to sit without any support and some of them could be stand, although they cannot walk.

• **Type III** (OMIM 253400): named Wohlfart-Kugelberg-Welander disease, is the mild chronic juvenile onset form. Patients reach the

ability to walk unaided, and some of them could never need wheelchair assistance during childhood.

• **Type IV** (OMIM 271150): is the form that presents in adulthood. Patients have the ability to walk and no problems related with respiration and nutrition are observed.

1.2 GENETIC AND MOLECULAR FINDINGS

For the first time, in 1990, the causative gene of SMA was mapped in a complex region of chromosome 5q (5q11.2-13.2) which contains an inverted duplication [7, 11]. In 1995, the Survival Motor Neuron (*SMN*) gene, which is located in that region, was identified as responsible for the disease[3, 12, 13].In the human genome, there are two almost identical *SMN* gene on chromosome 5q13: the telomeric or *SMN1* gene, which is the SMA causative gene, and the centromeric one or *SMN2* (**Figure 1**).

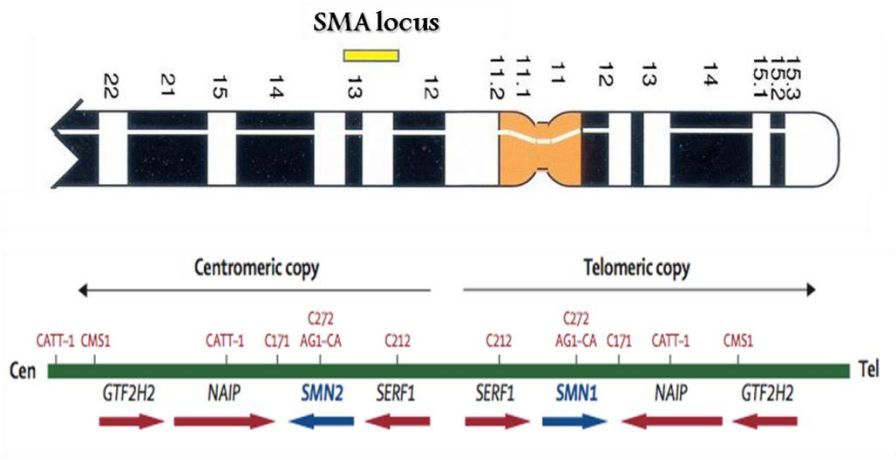

Figure 1: Locus of SMA causative gene, localized in a complex region of chromosome 5q (5q11.2-13.2).

This region may be subjected to intrachromosomal rearrangements as gene duplication, conversion or de novo deletions. Homozygous *SMN1* deletions of exons 6-8 are the usually involved mutations; however, frameshift, missense and nonsense *SMN1* variants have also been proved as responsible for SMA [14].

1.2.1 *SMN* GENE

The *SMN* gene is composed of nine exons (exon 1, 2a, 2b, 3-8) and eight introns and it covers a genomic region of about 20 kb with the stop codon located at the end of exon 7. The difference between the sequence of the two genes is the substitution of only 5 nucleotides (**Figure 2**), of which one is located in the coding region of the gene [15].

The *SMN*1 gene encodes a homonymous 38 KDa protein of 294 aminoacids which is ubiquitously expressed in human somatic tissues. While *SMN*1 gene is highly conserved in species from yeast to man, *SMN*2 gene is only found in the human genome [16].

SMN is required for life considering the fact that every patient has an homozygous mutation, rearrangement or deletion in the *SMN*1 gene, but at least one copy of *SMN*2 gene [17]. The severity of the disease phenotype is mainly related to the number of copies of *SMN2* that each patient retains. Two copies of *SMN2* usually determine SMA type I [18], three copies determine SMA type II and three or four copies of *SMN*2 determine SMA type III or IV[19, 20]. Patients with more than 5 copies of *SMN*2 show no sign of SMA even if they lack *SMN*1 [21]. In fact, about 10% of full length transcripts are produced by every copy of *SMN*2, and consequently the increased expression of protein SMN-FL is due to *SMN*2 gene, which is a benefit for patients.

From a molecular point of view, *SMN*2 is subjected to a particular process called alternative splicing, which occurs during the transcription. The alternative splicing of SMN2 pre-mRNA produces an mRNA lacking of exon 7 or, rarely, exon 5 or both exons. The exclusion of exon 7 from the mRNA of *SMN*2 is caused by a single C>T substitution in position +6 of this exon [22]. Despite being a silent mutation, which does not alter the amino acid sequence of the translated protein, it is localized at the level of an exonic splicing enhancer and therefore removes exon 7 during the transcription. In 2002, Cartegni and Kranier supported the hypothesis that the substitution C>T in exon 7 alters the sequence of an Exonic Splicing Enhancer (**ESEs**), suppressing its function. The exon 7 of the *SMN* gene is characterized by a weak splice site 3'. The inclusion of exon 7 in mRNA is positively regulated by many exonic factors in cis, called intronic splicing enhancer, while it is negative regulated by exonic elements in cis called Exonic Splicing Silencers (**ESSs**) or Intronic Silencers (**ISSs**). All of these cis elements (**Figure 2**) are recognized by transacting splicing proteins rich in serine-arginine (SR proteins) and several heterogeneous nuclear ribonucleoprotein (hnRNPs) [23].

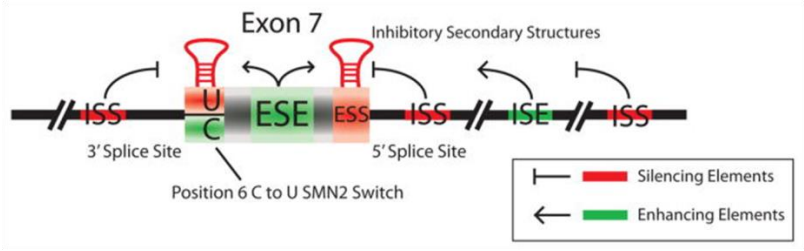

Figure 2: Splicing architecture of exon 7 of *SMN* gene: multiple RNA elements influence alternative exon 7 recognition.

In exon 7, the exonic splicing enhancer binds the Splicing Factor 2 (SF2), also named alternative splicing factor (ASF). SF2, in order to

form the splicing complex involved in the intron 7 removal from *SMN1* pre-mRNA, binds in turn the U2 class of small nuclear ribonuclear protein (U2 snRNP) and the U2 auxiliar factor (U2AF).

A second hypothesis proposed in 2003 by Kashima and Manley argued that the substitution C > T in *SMN*2 generates an ESS that interacting with hnRNP A1 facilitates the exon 7 skipping. This theory is supported by the knockdown of hnRNP A1 by siRNA (small interfering RNA) which induces an increase in exon 7 inclusion [24]. These two models are not necessarily incompatible and a third hypothesis, joining the two above-mentioned mechanisms, explain the alternative splicing of *SMN* transcript in a dual mechanism that includes both the loss of a specific ESE for SF2/ASF and the simultaneous creation of a specific ESS for hnRNP A1 [25]. The most important intronic splicing regulator of introns 6 and 7, is ISS- N1 which exerts a powerful effect on the activity of other positive elements in cis of exon 7 and intron 7. Antisense oligonucleotides against ISS- N1 determine the inclusion of exon 7 in the majority of *SMN2* mRNA transcripts [26].

According to another study, an exonic splicing suppressor [25] promotes the binding of heterogeneous nuclear ribonuclear protein A1 (hnRNP A1) or splicing suppressor protein, and sterically obstruct the formation of the splicing complex or its stabilization [24]. Even if the two hypotheses have been tested, the exact mechanism of the exon 7 exclusion remains an unsolved question (**Figure 3**).

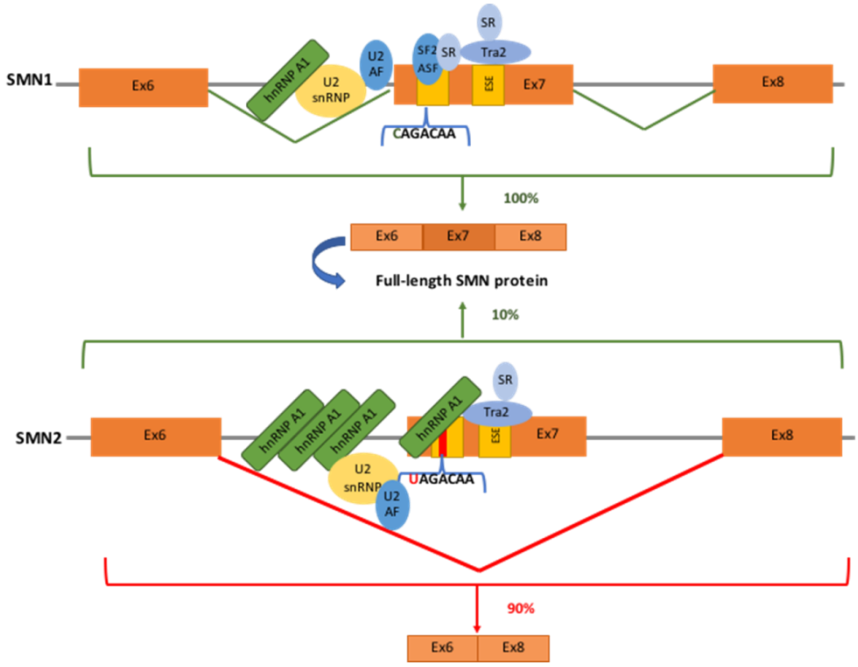

Figure 3: Alternative splicing process of the *SMN1* and *SMN2* pre-mRNAs. The C6T substitution within *SMN*2 causes the removal of exon7 from about 90% of the *SMN2* mRNAs

A greater understanding of this mechanism will surely lead to a significant progress in the possible therapeutic approaches for SMA disease.

Despite the different mechanism, *SMN*1 gene produces a full-length (FL) transcript that undergoes to a correct splicing in almost 100% of cases, while *SMN*2 produces only 10% of the transcript FL and in 90% of cases produces a transcript missing exon 7 (SMNΔ7). The full length mRNA *SMN1* and *SMN2* genes coding for identical proteins of 294 amino acids, while the transcript missing exon 7 (isoform SMNΔ7) encoding for a truncated protein of 282 aa which is unstable and has a reduced ability to oligomerization; this results in a lower functionality of the protein and an early degradation [27] (**Figure 4**).

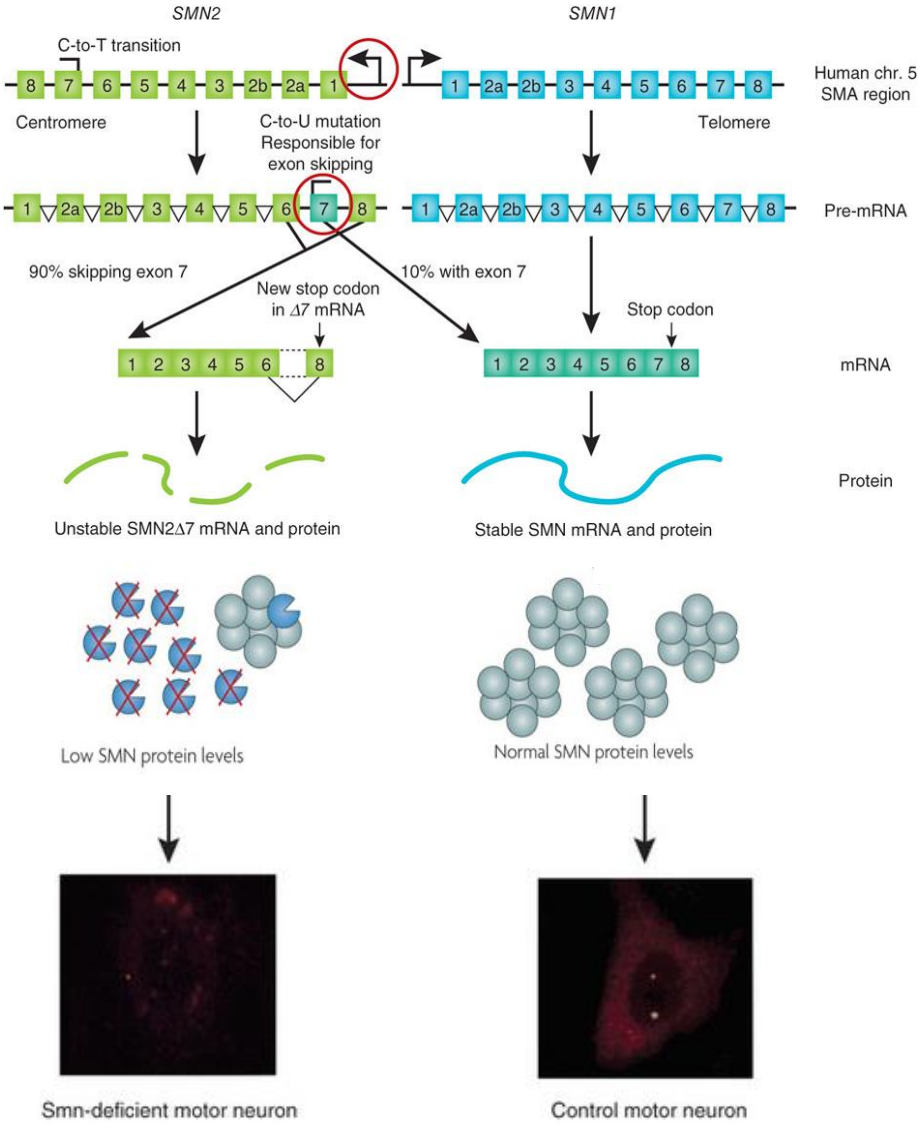

Figure 4: The Survival Motor Neuron Genes *SMN*1 and *SMN*2 have an identical structure and are 99.9% identical at the sequence level. The difference between the two genes is a single-nucleotide change in exon 7 (C or T). This single nucleotide change affects the splicing of the gene, so most SMN transcripts from *SMN*2 lack exon 7, whereas those from *SMN*1contain exon 7. The loss of the amino acids that are encoded by exon 7 results in the production of an SMN protein with severely decreased oligomerization efficiency and stability, and the *SMN* monomers are rapidly degraded. In a minority of cases, SMN2 transcripts contain exon 7 and encode for a normal, full-length SMN protein.

SMN2 gene produces only a small fraction of functional SMN protein than its homologous telomeric counterpart, insufficient for normal survival of motor neurons [28, 29].

1.2.2 SMN PROTEIN

SMN genes encode for the evolutionarily conserved and ubiquitously expressed 38 KDa SMN protein. This protein, which is particularly abundant in motor neurons of the spinal cord, is localized in the nucleus and in the cytoplasm of cells. There are particular structures inside the nucleus of the cells through which SMN protein is visible in association with coiled bodies (or Cajal bodies). These structures are called Gems (Gemini of coiled bodies) and their presence is a mark for cells of healthy patients, while in cells of patients affected by SMA their number decreases [30].

SMN forms a complex with gemini 2-8 and regulates the assembly of small nuclear ribonuclear proteins (snRNPs) [31, 32]. snRNPs are complexes of RNA-proteins that take part in the formation of the spliceosome.

The spliceasome, which is responsible for the splicing of the new synthetized pre-mRNA, is very active during the embryonic and post-natal development, while it is less active when myofibers and neurons are fully differentiated [33].

There are two main hypotheses to link the lack of SMN protein and the MN degeneration. The first one involves the impairment of the snRNPs complex. According to this hypothesis, when the normal splicing does not occur, the functions and survival of MNs are compromised. It has been demonstrated that when the levels of SMN protein are low, there is a lack of U11 and U12 snRNPs which constitute the minor spliceasome, responsible for the splicing of a minority of introns [34].

Some of them are mainly expressed in the nervous system and encode for essential neuronal component, like synaptic components and voltage gated ion channels.

The second hypothesis is based on the specific role that SMN protein could have for MNs, such as mRNA transport along the axons [35]. Furthermore, SMN protein seems to be involved in the membrane remodeling of MNs, which is very specialized for its extreme polarity, for the extension of the cellular processes and for the presence of neuromuscular junctions (NMJs). These data suggest that *SMN* is involved in MNs function and viability through the regulation of axonal transport, the remodeling of membrane domains and the preservation of NMJs' structural activity.

1.2.3 ANIMAL MODELS OF SMA

In order to understand the causes and the pathological hallmarks of the disease and to develop therapeutic strategies for clinical trials in humans, the use of animal models is fundamental.

So far, the models of SMA have been obtained in different species.

One of these is Drosophila, in which the human mild form of SMA has been reproduced, through a point mutation in the *SMN*1 gene [36]. Due to the high reproducibility of the animal, this model is very useful for a large scale screening of therapeutic molecules.

Another animal model used is Zebrafish [37]. When the *SMN* gene is downregulated through antisense oligonucleotides (ASOs), it is possible to observe neural structural abnormalities and MN apoptosis of MNs, similar to those found in humans. Thanks to the easy manipulation and the transparency of its body, Zebrafish is very useful for the study of *SMN* in pre-natal and post-natal development, and for the first step for large scale *in vivo* drug screening [37].

1.2.3.1 MURINE TRANSGENIC MODELS

The murine model represents the model closer to the human pathology and more useful to the clinical research. Mice harbor a single copy of the *SMN* gene. If *SMN* gene is disrupted in homozygosis, there is a failure of early development of mice embryos and pre-natal death [38].

Different transgenic SMN -/- mice have been generated, carrying one or more copies of the human *SMN*2 gene (**Table 2**). The severity of their phenotype is inversely correlated with the number of *SMN*2 copies.

The most commonly used murine models are:

- *Smn1-/-; SMN2tg/tg; SMNΔ7tg/tg* (usually identified as SMAΔ7 mouse) [39]
- *Smn1hung-/-;SMN2Hungtg/tg* (Hung-Li model) [40]

The first available model of type II SMA was the Hung-Li model. It has a homozygous null mutation in the murine *SMN* gene and harbours a full-length human *SMN*2 transgene.

After another model was developed by Monani et al. that resembles SMA type I, carrying a homozygous null mutation in the *SMN* gene and the human *SMN*2 transgene [41]. The signs of the pathology are a motor impairment from post-natal day 2 (P2), the loss of about 40% of lower MNs and the death around P5.

The same investigators created another transgenic mouse model showing an intermediate SMA type I/II phenotype, adding the cDNA of the human *SMN*2 lacking exon 7 to the previous model [39].

This model starts to present signs of muscle weakness at P5, loses about a half of lower MNs and dies at 13 days of age. Before the decline of motor functions, the first alteration is the structural abnormality of NMJs, which are not completely mature and are not able to cluster the acetylcholine receptors.

Another mouse model developed by Monani et al., called SMN1A2G, resembles SMA type III in humans because it carries a *SMN*1 transgene with A2G missense mutation [42].

Despite all these mouse models, SMA∆7 is the one which is commonly used because it reproduces better the human SMA phenotypic characteristics.

The only issue related to these mice is the limited lifespan which not allow to evaluate some therapeutic approaches.

Model Name	Genotype	SMA type	Life expectancy	Phenotype
Smn1 null mutation	*Smn -/-*	Type I	Pre-natal mortality	Severe
Hung-Li mouse	*Smn1$^{hung-/-}$;SMN2Hung$^{tg/-}$*	Type II	13 days	Intermediate: Similar to the ∆7 mouse model.
∆7 mouse model	*Smn1$^{-/-}$; SMN2$^{tg/tg}$;SMNΔ 7tg/tg*	Type II	13 days	Intermediate: Muscle weakness is present from P5 and progress over time, abnormal gait, tendency to fall. ByP9 around 50% of LMNs are lost.
A2G mouse	*Smn1$^{-/-}$; SMN2$^{tg/tg}$;SMN1 A2G*	Type III	Less than 1 year	Mild: Milder than the Δ7 mouse. Decreased body weight, muscle atrophy and weakness.

Table 2: commonly used SMA murine models

1.3 DIAGNOSIS

1.3.1 GENETIC COUNSELING AND PRENATAL DIAGNOSIS

The screening test generally used to detect SMA is a semi quantitative real time PCR or MLPA, recognizing the heterozygous deletion of one *SMN1* allele, as the most frequent mutation is the homozygous absence of *SMN*1 gene.

There are some limits of the prenatal diagnosis process that cannot establish an accurate prognosis, indeed the severity of the potential affected fetus might not be predict a priori and the *SMN2* number of copies is not sufficient to establish an accurate prognosis.

The importance of the prenatal diagnosis is to identify affected infants before the onset of clinical symptoms. Even if a number of potential therapies are in clinical trials [43, 44], their success depends on the early identification of affected subjects, in order to administer the treatment before potentially irreversible neuronal loss.

1.3.2 MOLECULAR DIAGNOSIS

When disease phenotype is very severe, the diagnosis of SMA can be highly suspected from clinical features.

The first step for the molecular diagnosis is the detection of a homozygous deletion in *SMN1* [5]. A Restriction Fragment Length Polymorphism (PCR-RFLP) test is performed to analyze the absence of *SMN1* exon 7 (with or without deletion of exon 8). The sensitivity of this analysis is 95% but it is not able to identify carrier subjects. If this first test is negative, some other laboratory exams are performed.

Generally, when a single *SMN1* copy was found in a patient, coding region of the undeleted allele is sequenced to identify the second causative mutation, generally point mutations, insertions and deletions.

It has been observed that one third of patients with a single *SM*N1 copy and a typical phenotype of the disease does not show the second mutation in *SMN1/SMN2* coding region, especially in mild form of SMA (SMA III). The explanation could be found in the presence of deep intronic mutations, which are so far unpublished.

Usually when patients are born from consanguineous parents and have a typical clinical picture, are highly recommended to have a sequence

analysis of *SMN1* gene, while, in patients with 2 *SMN1* copies, other neuron disorders should be considered and SMA diagnosis related to *SMN1* mutations is virtually excluded.

1.4 THERAPEUTIC STRATEGIES FOR SMA

The discovery of molecular basis of SMA was a critical step to develop possible different therapeutic approaches [45]. Furthermore, the availability of animal models showed a correlation between the disease and the low expression of *SMN* gene. There are different approaches used as therapeutics for SMA and many of them are under evaluation in human clinical trials (**Figure 5**).

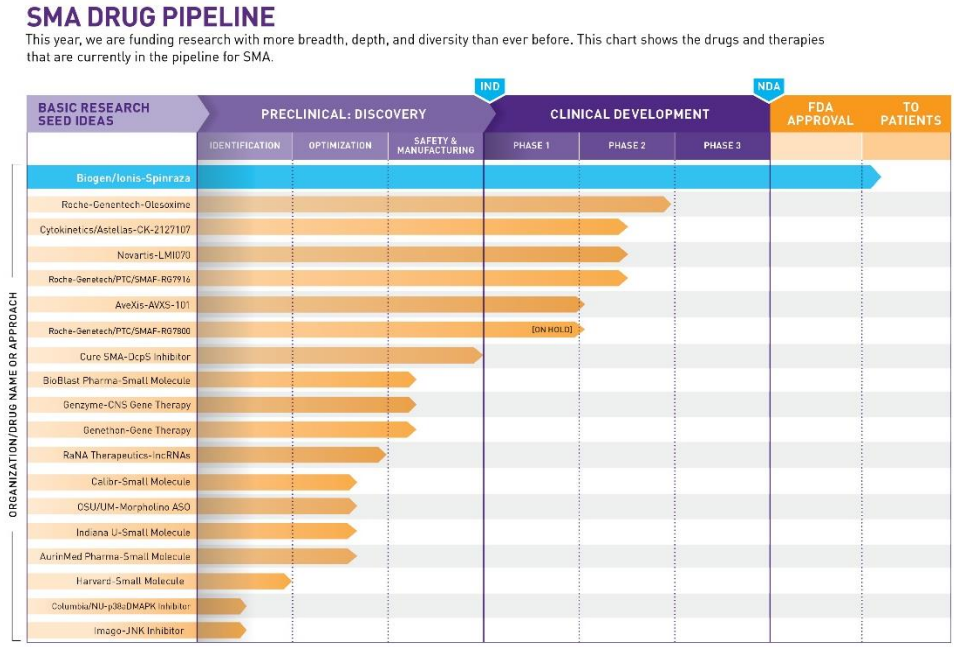

Figure 5: Current status of investigational drugs for SMA. Modified from Curesma.org

These approaches are based on different techniques such as the replacement of the mutated *SMN1* gene, the modulation of *SMN2* splicing and expression or the protection of MNs from degeneration.

On December 2016, the first therapy for SMA based on the splicing correction, named Spinraza, has been approved by FDA, followed in April 2017 by the EMA approval. The success of this approach testified the importance of translational medicine.

Overall the main investigation fields for SMA treatment can be classified into three groups:

1.4.1 GENE THERAPY

Gene therapy is based on the use of different viral vectors to transfer a functional copy of the *SMN1* gene into patient's cells, specifically into MNs.

There are two strategies currently used as gene therapies: the replacement of *SMN1* [46] and the augmentation of *SMN2* read-through. The aim of this therapy is to restore a normal form of the *SMN1* gene in SMA patients, although the most challenging step is the delivery to a difficult-to-access cells, such as motor neurons.

There is a particular viral vector with a specific tropism for the cells of CNS, the adeno-associated viral vectors (AAV) [47, 48]. The peculiar characteristic of these vectors is the ability to transduce non-dividing cells and to give long-term stable gene expression in order to increase the levels of SMN protein.

In 2010 Kaspar group demonstrated that systemically delivered AAV9-SMN delivered early to a severe rodent model of SMA7 results in substantial rescue of the lifespan. This work was published in Nature Biotechnology in 2010, as well as similar reports followed [49].

In 2010, after the administration with an intrathecal injection of self-complementary (sc) adeno-associated virus serotype 8 (scAAV8) encoding a wild type copy of the human *SMN1* gene in SMA mice, Passini and colleagues reported an increase of the lifespan of the treated mice [50].

Moreover, the research group of Dominguez reported a rescue of motor function and NMJ physiology after the *SMN1* delivery through scAAV9. The administration of the vector was done through an intravenous (IV) injection, and the results obtained proved that it was able to reach the CNS and MNs. The treated mice had a prolonged life with a mean survival of 200 days, compared to 13 days of the untreated mice [51].This vector was able to cross the BBB also in non-human primates, confirming its efficacy in large animals and humans [52].

Gene therapy has been recently evaluated in a phase 1/2 clinical trial, called AVXS-101 (a sc AAV9 carrying the *SMN* gene), on SMA type I subjects[1].The preliminary analysis of the results of this trial are very promising. The survival rate of the enrolled patients increased of 100% at 20 months, and the motor performances improved. Some infants were able to sit without support, but the most positive result was achieved by two of the twelve infants who were treated with the highest dose of the vector: they are able to walk independently.

Given these promising results, AVXS-101 was granted access to the EMA PRIME Program for SMA type I in January 2017[2]. The preliminary data disclosed by AveXis offer positive expectation for the progress of gene therapy to more advanced phase trials and its clinical successful application.

[1] ClinicalTrial.gov Identifier: NCT02122952

[2] AveXis, Inc. http://investors.avexis.com/

1.4.2STEM CELLS THERAPY

Another treatment investigated in SMA is the trophic support in CNS.

One of this strategy is based on neural stem/precursors cell transplantation. The first step of this approach is the differentiation of Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) into neural stem cells (NSCs). NSC transplantation has been tested in severe SMA mice and positive results in terms of increased survival and improved motor function have been reported [53].

Some properties of iPSCs for stem cell therapy make their use more convenient, rather than ESCs. First of all, their generation from the patient's somatic cells avoids the eventual risk of rejection after transplantation. Then, they can be edited genetically to correct the SMN1 mutation [54]. IPSCs can be generated using non-viral and non-integrating episomal vectors, that are lost over time, eliminating exogenous genetic sequences from the reprogrammed cells.

In order to ameliorate the SMA mice survival and phenotype, it has been proved that the release of growth factors by transplanted iPSCs derived NSCs is the principal mechanism underlying their beneficial effect.

All these positive pre-clinical results suggest that this technique warrants further study.

1.4.3 PHARMACOLOGICAL TREATMENT

1.4.3.1 ENHANCEMENT OF SMN2 GENE EXPRESSION

There are some factors that influence the transcriptional activity of *SMN2*, such as CpG islands methylation of histones. Some compounds have been tested in order to enhance *SMN2* transcription, like inhibitors of the histone deacetylase enzyme (HDACi) and short-chain fatty acids.

All these drugs were efficient *in vitro* but they did not show the same positive results *in vivo* [44, 55-57], probably due to the fact that they are active in replicating cells, but not in many mature cells like neurons.

Another possible therapeutic approach that has been investigated is the inhibition of protein degradation through the proteasome. A proteasome inhibitor, tested in SMA mice, is bortezomib. It has been showed that after its administration, SMA mice improved their muscular functions but not their survival [58], because bortezomib could not cross the BBB it could not act on MNs [59]. These results highlight the necessity of a drug to cross the BBB and reach the CNS in order to improve the SMA mice survival and phenotype.

A positive result in terms of increased amount of SMN protein in *in vitro* and *in vivo* studies has been obtained with quinazoline derivatives, which enhance the activity of *SMN2* promoter [60].

1.4.4 SMALL MOLECULES

The goal of the small molecules is the modulation of the alternative splicing of the *SMN2* gene in order to produce a full-length SMN protein. Currently some of them are tested in clinical trials. In particular, Roche-Genentech/PTC Therapeutics and Novartis developed some molecules that have entered Phase II clinical trial RG7916 and LMI1070 respectively.

RG7916 is an oral bioavailable small molecule which in preclinical studies showed an improved motor function and survival up to more than 150 day in SMA mice [61]. On October 2016, the Phase II clinical

trials started on patients with type II and II SMA[3], while on January 2017, a Phase II clinical trial started on patients with SMA I[4].

LMI1070 is able to enhance exon 7 retention in the SMN2 mRNA, stabilizing the transient double strand RNA structure formed by the *SMN2* pre-mRNA and U1 small nuclear ribonucleic protein (snRNP) complex. It is currently tested in an open-label, Phase II clinical trial on infant with type I SMA[5].

1.4.5 ANTISENSE OLIGONUCLEOTIDES

Another therapeutic strategy to increase exon 7 inclusion is the use of Antisense oligonucleotides (ASOs), short modified oligonucleotides which bind specific mRNA sequences (**Figure 6**).

[3] PTC Therapeutics press release, available at: http://ir.ptcbio.com/releasedetail.cfm?ReleaseID=994745

[4] PTC Therapeutics press release, available at: http://ir.ptcbio.com/releasedetail.cfm?ReleaseID=1006620

[5] ClinicalTrials.gov Identifier: NCT02268552

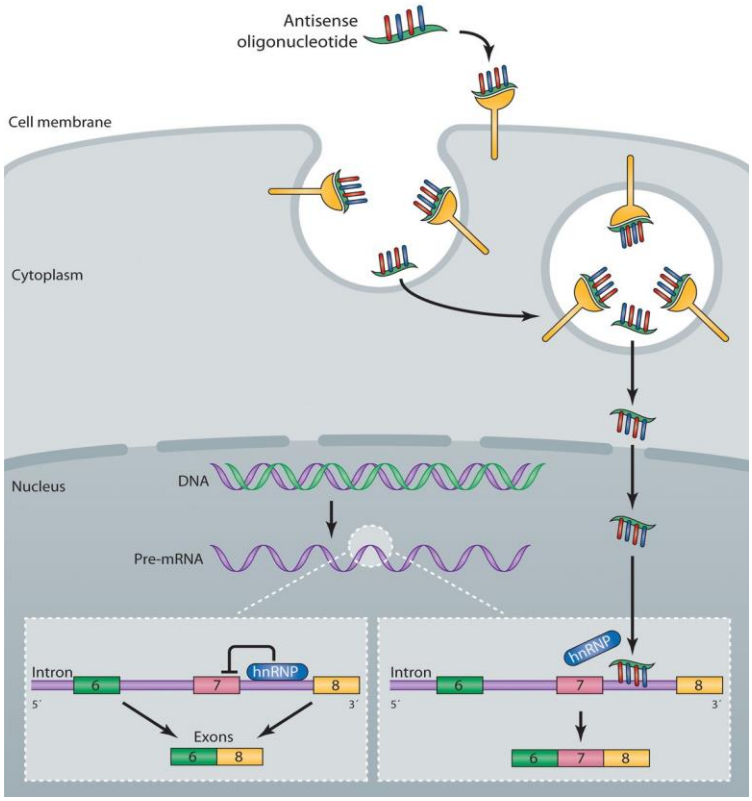

Figure 6: Cellular internalization and steric block action of ASOs

Different sequences of ASOs have been created to specifically target the intron splicing silencer (ISS) motif in the 5' of intron 7 in *SMN2* mRNA [26, 62, 63]. Several ASOs with different chemical structures have been tested in *in vitro* and *in vivo* models, with a particular attention to the modifications that enhance some biological properties, like the stability and resistance to metabolic degradation in biological tissues and fluids, the toxicity and the high ability to reach the target cells, comprising neurons within the CNS.

The three different ASOs chemistries tested are:

- 2'-O-methyl phosphorothioate (2OMePS)
- 2'-O-Methoxyethyl (MOE)
- Morpholino (PMO)

The most efficient approach is the combination of a specific ASO sequence with an oligomer that binds positive splice activators. Several investigators showed that ASOs targeting the ESE altered by the C6T substitution within the *SMN2* gene enhanced the production of full-length SMN *in vitro* [64]. This discovery was followed by a series of preclinical studies using 2OMePS ASO[65-73].

In order to promote a strong exon retention, chemically modified ASOs (MOE) that are resistant to endonuclease activity and are not dependent on RNase-H degradation have been used. ISS-N1 sequence is so far the most important splice modulator adjacent to the exon 7 5'ss, involved in SMA studies.

Several data indicated that the dosage and the early starting of the treatment influence the efficacy of ASO mediated therapy [68]. It has been confirmed that if SMA mice were treated with a low dose of MOE ASO with ICV injections, there was a poor effect on their survival [74]. There was an increase of the lifespan up to more than 500 days when a higher dose of MOE has been administered via multiple subcutaneous (SC) and intraperitoneal (IP) injections. Obviously, the most relevant problem connected to the use of ASOs, such as the limited capacity to cross the BBB, caused a significant increase of SMN protein levels in the peripheral tissues.

Another important aspect to be evaluated is the safety profile of 2OMePS and MOE ASOs. The second one was shown to be the safer one, while the first one caused an increase in inflammation in the spinal cord and a lower increase of SMN levels after the delivery into the CNS [75].

The proof that MOE ASOs are efficient and safe molecules, makes them suitable for human clinical trials. In fact, a MOE ASO (named Nusinersen, commercial name Spinraza) has been developed by Ionis

pharmaceutical. Administered by a lumbar puncture, it reaches the CNS and targets the ISS-N1 region [76, 77].

Ionis Pharmaceutical and Biogen started a collaboration for studies in SMA patients. The clinical study program of Nusinersen consisted in two Phase III, double blind, controlled trials, identified as ENDEAR and CHERISH, and two additional Phase II controlled studies, called EMBRACE and NURTURE.

The ENDEAR study investigated the effect of Nusinersen on SMA type I patients with the first treatment at 6 (or less) month old, while Cherish study was focused on SMA II patients between 2 and 12 years old at the time of screening. On November 2016, CHERISH study met its primary endpoint and treated patients reached an increase of 4 points at the Hammersmith Functional Motor Scale Expanded (HFMSE) respect to a decline of about 2 point of non-treated subjects. The EMBRACE study was conducted on SMA I and II patients who did not satisfy the criteria to be enrolled in ENDEAR[6] and CHERISH[7] studies. The NURTURE study is an open label trial performed on presymptomatic infants. None of the patients showed adverse effects even if the results of the Interim analysis of the NURTURE reached a motor milestone closer to the ones of untreated patients.

On December 2016, Nusinersen (named Spinraza) was approved by the U.S. FDA as the first therapy for SMA[8]. The EMBRACE and NURTURE studies will provide knowledge about the therapeutic window to obtain the maximum therapeutic efficacy.

[6] Biogen: http://media.biogen.com/press-release
[7] Biogen: http://media.biogen.com/press-release
[8] Biogen: http://media.biogen.com/press-release

1.4.5.1 MORPHOLINO

A particular type of ASO is Morpholino (MO) in which the phosphorothioateribose backbone is replaced by phosphorodiamidate-linked morpholine backbone that is refractory to metabolic degradation (**Figure 7**).

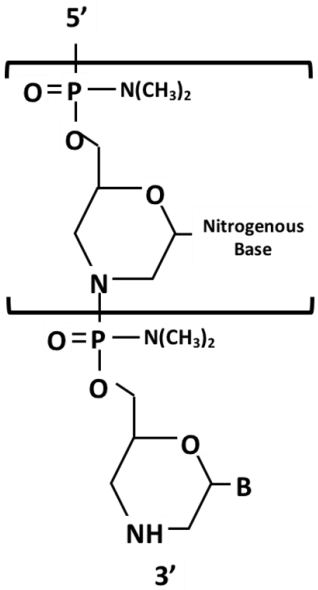

Figure 7: Chemical structure of MO ASOs

The low toxicity of this compound has been proved in preclinical studies and its ability to not elicit the immune response makes it interesting for in vivo uses, in particular in clinical trials, such as what was already tested for Duchenne Muscular Dystrophy [78, 79].

These characteristics make it a suitable candidate for molecular therapy in SMA. However, there are several issues to be addressed, such as its sequence and the modalities of its administration. In fact, it is not clear if a local injection is sufficient to rescue the SMA phenotype [80] or if a systemic injection is necessary and sufficient [74].

The MO's mechanism of action is based on a steric block, that hampers the mRNA translation. The length of MO oligomers is crucial for their

function. Burghes and colleagues tested MOs with different length (20,22 and 25 mers) and they demonstrated that the MOs' length is directly correlated to the efficiency in human SMA fibroblast [81]. Another characteristic that influences the efficacy of MO is the position of the target sequence within ISS-N1. The most consistent effect was achieved when targeting 8-35 base region of intron 7 [82].Once reached the optimal length, the same group conducted dose escalation studies and showed that the optimal length is 25 mer (MO-10-34) because this MO can exert its action even when it is administered at lower doses.

Other *in vitro* studies confirmed that MO-10-34 is the most efficacious sequence and for this reason it has been tested *in vivo*. When homozygous SMA-Δ7 mice were treated with MO-10-34, a splice-correction was observed and the mean survival was extended (>120 days) [82].

The big challenge of this strategy is to avoid the ICV administration, necessary because of the scarce bioavailability and concentration of MO in the CNS after the systemic injection. For this reason, our group evaluated the efficacy of MO-10-34 sequence, synthetized as unmodified MO oligomers (bare-MO) or octaguanidine-conjugated MO (Vivo-MO) to test their efficacy on survival and motor function of SMA mice [83]. The results obtained showed that Vivo-MO is capable to cross the BBB thanks to its guanidinium group, respect to bare MO. After the treatment of SMAΔ7 pups with systemic and local injections, we determined that Vivo-MO is as efficacious as bare-MO but more toxic, limiting the maximum dose achievable. Moreover, the combined treatment of intracerebroventricular (ICV) and subcutaneous (SC) injections at the maximum dose of 12 nmoles per injection, improved mice survival and motor function respect to lower doses and to the local

or systemic injections alone. Our results suggested that if there is an increase of SMN protein levels in tissues outside the CNS, it could improve the motor performance and the survival rate and the optimization of MO sequence with its systemic delivery are crucial issues to optimize this therapeutic strategy.

1.5 DRUG DELIVERY TO THE CENTRAL NERVOUS SYSTEM

One of the limiting steps for the systemic delivery of a drug in the treatment of neurological disease is reaching the CNS. The CNS has innate physiological barriers that protect it from toxins and infections. These barriers are the BBB and the blood-cerebrospinal fluid barrier (BCSFB).

The main characteristic of this area is that the lipophilic molecules with a molecular weight of maximum 600 Da can pass through it by free diffusion, while the larger or hydrophilic molecules are transported with active systems, by carriers or receptors[84].

The positively charged molecules can enter the CNS by an endocytic pathway, called absorptive-mediated transport [85].

The highly selectivity of this barrier has a double effect: if from one side it protects and supports the function the CNS, on the other side it is a challenging obstacle to drug delivery. This is the reason why the proper design of drugs is a very important step in the treatment of neurological diseases.

1.5.1 CROSSING THE BLOOD-BRAIN BARRIER

The ability of a drug to cross the BBB depends on its physical and chemical properties, like size, structure, lipophilicity, molecular weight, volume of distribution and affinity for receptors or efflux pumps. Some

approaches to cross the BBB in a non-invasive way could be the generation of:

- chimeric peptides
- viral vectors
- liposomes
- cell-penetrating peptides (CPPs)
- nanoparticles (NPs)
- dendrimers.

We will discuss below the importance of CPPs in our study.

1.5.2 CELL-PENETRATING PEPTIDES

1.5.2.1 DEFINITION AND CLASSIFICATION

Some of the problems that prevent several therapeutic strategies to be efficiently translated to the clinic are the biodistribution, the bioavailability and the pharmacokinetic of the drugs/molecules used in neurological diseases. In fact, the design of molecules that can cross the BBB, reaching their target site, is a critical step in neurodegenerative disorders.

Over the last 20 years, more than 100 peptidic sequences of 5-40 amino acids have been identified to promote the drug delivery [86]. These peptides, also named as Cell-penetrating peptides (CPPs), can cross biological membranes and translocate into the intra-cellular compartment [87, 88]. Their main advantage is their ability to delivery different compounds, such as proteins [18, 19] nucleic acids, siRNA [89], nanoparticles [20] and ASOs, across biological barriers.

The CPPs have different physical-chemical properties and they can be divided in three major classes: cationic, amphipatic and hydrophobic. Most of them have a net positive charge (83%), anionic CPPs are assigned to different classes according to their basis, the amphipatic

CPPs form a large class (44%), and the hydrophobic are only 15 % [90, 91].

- Cationic class: this is the biggest class to which belongs the best-known CPP derived from HIV-1 protein TAT (TAT peptide). The arginine-rich sequences require a minimum amount of eight arginine residues named octaarginine (R8) for cellular uptake [92]. The ability of arginine residues to penetrate cells at physiological pH is an important characteristic for the transduction of these peptides. The ability of these peptides to cross the biological barriers has been developed by increasing the space between guanidinium groups and the backbone of synthetic peptides [93] or conferring a cyclic structure [94]. Moreover, these short cationic sequences develop electrostatic interactions with negatively charged glycoproteins on cellular surface and they can be used as transmembrane carriers for therapeutic compounds [87].
- Amphipathic class: in this class we can find peptides as MPG and Pep-1, or peptides derived from natural proteins as pVEC and secondary amphipathic α-helical CPPs in which hydrophobic and hydrophilic aminoacids are placed in different faces of the helix as MAP peptide and Transportan.
- Hydrophobic class: peptides based on natural aminoacids or chemically modified petides belong to this class. They seem to directly cross the cell membrane avoiding endosomal degradation.

Moreover, according to their origin, CPPs are classified as derived from natural proteins or synthetic polypeptides. To the first group belong the peptides that are able to pass through biological membranes. After this discover, chimeric peptides were designed in order to be used as biological carriers [95].

Artificial peptides are synthetized replacing natural amino-acids, generating chimeric sequences. They are classified according to their chemical structure and physical features.

Despite their classification, CPPs are very useful as a tool for drug delivery in neuromuscular disorders, being able to cross biological membranes and releasing therapeutics biomacromolecules in the target site (**Figure 8**).

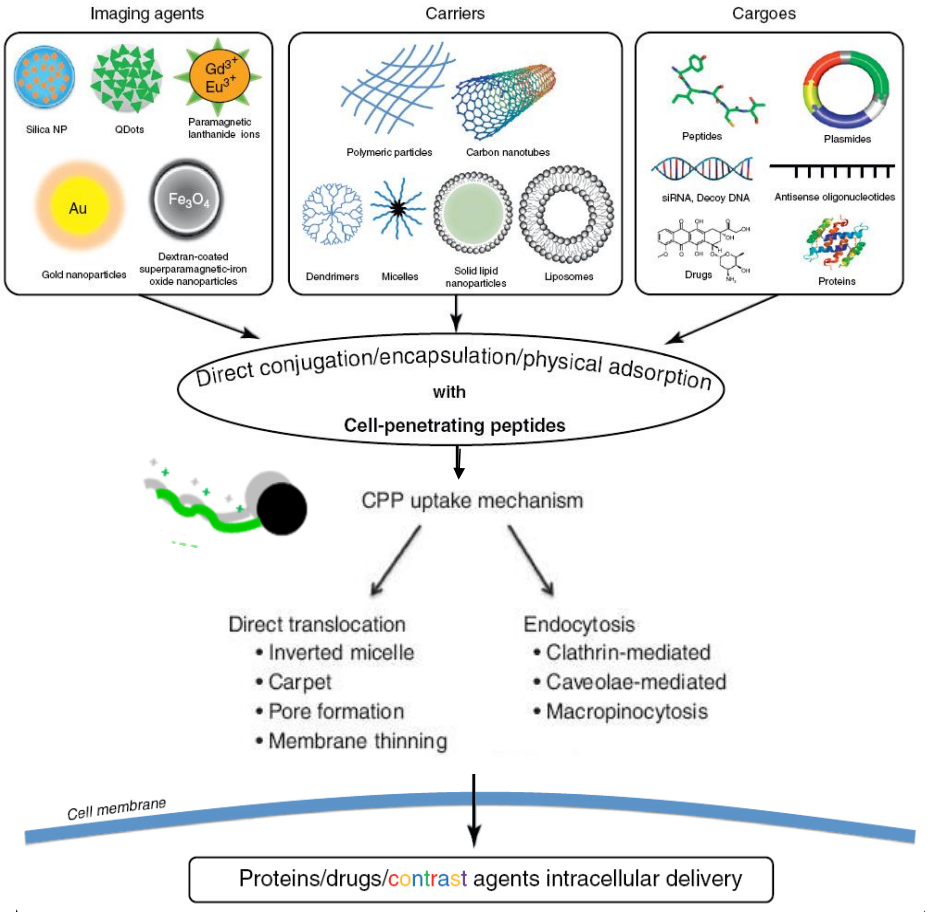

Figure 8: Cell-Penetrating Peptides. Applications as molecular delivery vehicles for a variety of drugs, nucleic acids, proteins, therapeutics and imaging agents and uptake mechanisms.

1.5.2.2 MECHANISMS OF INTERNALIZATION

Short, positive charged CPPs, interacting with the negatively charged BBB, increase cellular drug uptake and activate the permeabilization of cell membranes, stimulating the endocytosis of the cargo [96]. Endocytosis is a natural, energy dependent, cellular process which consists of electrostatic interactions with proteoglycans at cellular surface or involves direct destabilizing interactions across the lipid bilayer [90].

The peptides that constitute the CPPs interact with the proteoglicans in the cell membranes [97, 98] and this step is critical for the activation of a downstream signal pathway, inducing the pinocytosis [99].

Another mechanism used by CPPs to cross the membranes is called transduction. It is an energy independent pathway based on direct membrane penetration, using a pore formation model [90]. This mechanism of internalization requires a conjugation between the peptide and small molecules.

Even if different modalities of cell penetration can happen, endocytosis is the most important and clinically relevant one [100].

1.5.2.3 APPLICATIONS OF CPPs

In the recent years, CPPs have been used as a delivery tool taking advantage of their safety profile in *in vitro* and *in vivo* models. The principal applications of CPPs in the delivery of molecules of pharmacological relevance are following presented.

1.5.3 SMALL INTERFERING RNA (siRNA) DELIVERY

Despite being useful in the pharmacological field through gene silencing, siRNAs lack of a safe and efficient delivery that allow them to step into the clinical practice [101, 102]. When they are conjugated with

CPPs, they are successfully delivered both in *in vitro* and *in vivo* systems. There are different conjugation approaches: the simpler one consists on a non-covalent bonding between siRNAs and CPPs [89, 103]. The complexes obtained show a net positive charge. The use of oligo-arginine peptides reported good results in terms of siRNA delivery *in vitro* [104] and *in vivo* [105]. Another interesting strategy is the generation of a chimeric peptide composed by the protein transduction domain of the TAT peptide and a double strand RNA binding domain. This method tested by Eguchi et al.[106] has the advantage not to cause the activation of the immune system.

However, the better strategy seems to be the covalent bonding because it is easily reproducible and allows a precise stoichiometry of conjugates obtained and siRNA-CPP proportion [89].

The negative aspect of this technique is the higher concentration needed to achieve efficient gene silencing [107]. This situation was not observed when not purified siRNA-CPP complex have been used [108-110]. The reason has been found in the fact that the unpurified solutions are rich of covalently bound complexes, which can derive from the excess of siRNA and CPPs in the reaction [111]. Anyway, promising results were obtained with covalent bonding between siRNA and TAT [108], transportan [109] and penetratin [110].

There are several clinical trials ongoing or completed[9], but none of them seem to solve the bioavailability issue. Indeed, all these trials use saline-based solutions of siRNAs.

[9] Registered Clinical Trials were searched on www.ClinicalTrials.gov using the keywords smallinterfering RNA or the abbreviation siRNA.

1.5.4 DNA DELIVERY

An alternative of viral vectors for gene delivery is represented by CPPs [23]. Despite being widely used, viral vectors have some issues still unresolved [28]. After their use, we can observe toxicity, immunogenicity, and a restriction in the length of genes that can be packed into them. Another problem is the risk of inducing oncogenic mutation, as they can randomly be integrated into the host genome [28].

An alternative strategy for the gene delivery is the use of nanoparticles and liposomes [31, 45].

To date, CPP-based gene delivery has been evaluated *in vitro* and *in vivo*. Data showed good results of gene expression in both cell cultures and animal models [82, 112, 113]. In order to increase the transfection efficiency, different cationic CPPs have been chemically modified. Such modifications include stearic acid modification of arginine rich peptides TAT, REV, polyarginine with 4 to 16 amino acids, RxR4 [84, 85, 114].

Among them, TAT peptides produced controversial results. In fact, the lacking efficiency obtained *in vivo* did not correspond with the high one reported *in vitro*. The reason of this incongruity is probably caused by the poor *in vivo* stability of the CPPs and their poor resistance to metabolic degradation, due to the presence of serum proteins and peptidases.

To overcome this problem, one possible strategy is to use multivalent ramified structures, as reported by Lo et al. [115]. They used a structure harboring 1 to 8 TAT peptides with the addition of multiple histidine and cysteine residues that increased transfection efficiency [116].

Another good result has been obtained with the use of non-viral vector-CPP conjugates [117]. Some of them are made of CPPs linked to PEI,

PEG- PEI [118, 119],or liposomes [120] and non-covalent complex of DNA, liposomes and CPPs (lipoplex) [121, 122].

The efficiency of lipid vectors has been boosted with this approach in *in vivo* models. One positive aspect of this method is that CPPs allow the penetration of liposomes and their cargo into the cells, while liposomes protect the drug and keep it from the degradation until it reaches the cellular target.

1.5.5 ANTISENSE OLIGONUCLEOTIDES DELIVERY

The main issue that obstacles the use of ASOs in clinic is their poor bioavailability.

In 1998, Pooga et al. [123] demonstrated the efficacy of CPP-ASO conjugates *in vitro* and *in vivo*. In the following years, CPP-ASOs have been used to treat Duchenne Muscular Dystrophy (DMD) by an exon skipping approach [79]. In particular, several studies on DMD animal models showed that the best used CPPs were arginine-rich CPP such as (RXR)4 [124] and (RXRRBR)2 [125]. These conjugates were effective in inducing exon skipping *in vivo* and showed a dose-related response in dystrophin restoration. After the treatment, it has been observed an amelioration of the disease phenotype due to the presence of a functional dystrophin protein in several muscles.

Moreover, these studies did not report significant signs of toxicity.

In the last years, a new class of arginine-rich CPPs named “Pip” have been investigated in murine models of DMD [126-128]. The structure of these peptides is made of a hydrophobic core and the external faces are positively charged with arginine residues. The interesting result obtained after the use of these peptides is that ASO conjugated to Pip5e and Pip6a were highly effective when they are administered at very low doses. These data support the thesis of a considerable reduction of the required dose and of the ASO-induced toxicity.

The approach of CPP-MO oligomers has been successfully used to treat other kind of diseases and even viral or bacterial infections [129]. In particular, it has been improved the capacity of ASO to inhibit viral replication in vivo by blocking the transcription of viral genome [130-134]. As a matter of fact, to treat bacterial infections, different MOs with antimicrobial activity have been coupled with CPPs [135-138]. The advantages of this technique is the possibility to decrease the dosage of many antibiotics, and probably the toxicity of many of them, and the increase of bacterial membrane permeability to several antibacterial compounds.

The conjugation of MOs with CPPs can occur either via covalent or non-covalent bonding [139, 140]. The strategy of covalent conjugation seems to be better because it is very reproducible and it allows to obtain a precise stoichiometry of the synthetized construct, while the synthesis of non-covalent complexes may alter the biological activity of the associates cargos.

1.5.6 PROTEIN DELIVERY

Another class of molecules that can be used as therapy in many diseases is represented by proteins. But there are a lot of issues linked to their use. First of all, in order to preserve their biological activity, they have to maintain their tertiary or quaternary structure. The final structure is guaranteed by weak and non-covalent bonds between amino acids residues and this is the reason of short half-life, poor penetration across biological membranes and low stability of proteins in biological fluids and tissues.

To overcome this problem, it has been tested the use of CPPs to deliver therapeutic proteins *in vivo*. Several proteins such as human catalase, human glutamate dehydrogenase [141], Bcl-xL [142], Cu/Zn SOD [143], heat shock protein 70 [144], the Nf-kB inhibitor srIkBa [145],

eGFP [146] are some examples of how this strategy has been successfully employed.

The CPPs-protein conjugates can be synthesized using a covalent or non-covalent bonding [147, 148], as previously reported for siRNAs, oligonucleotides and DNAs, or it is possible to generate a single chimeric protein without the need of additional modification.

1.6 CLINICAL TRIALS

Ended before completion date, the first Phase II clinical trial was based on the use of CPP-cargo in humans and was conducted in 2003 as a treatment for psoriatic lesions (PsorBan by CellGate Inc.).

Another Phase II clinical study, completed in 2010 (NCT00451256), was named AVI-5126. The aim of the CPP-MO conjugate was the reduction of the occurrence of coronary artery restenosis by inhibition of c-myc. This trial was sponsored by Sarepta Therapeutics, which is also developing other conjugates. One of these is AVI-5038, an (RXRRBR)2 XB-MO construct used to treat Duchenne Muscular Dystrophy [149]. The mechanism of action of this compound is the exon 51 skipping in the dystrophin gene, inducing the expression of a shorter but functional dystrophin protein. Even if AVI-5038 is under preclinical evaluation, it has been expected to be enter clinical practice soon as an unconjugated MO oligonucleotide AVI-4658 (called Eteplirsen)[10].

In order to reduce the area of myocardial damage after acute myocardial infarction, the drug KAI-9083 has been tested in a human Phase II clinical trial [11]. It is a conjugate of the selective inhibitor of the

[10]Source:http://investorrelations.sarepta.com/phoenix.zhtml?c=64231&p=irolnewsArticle&ID=2204492

[11] Registered on ClinicalTrials.gov, Identifier: NCT 00785954

d-Protein Kinase C (d-PKC) with the TAT peptide. The trial ended in 2011 and didn't demonstrate the efficacy of the compound. However, the trial proved the safety of the compound also in humans.

Another drug, similar to KAI-1678, a g-PKC-TAT conjugate, have been used to reduce the pain in spinal cord injured patients. It has been tested in a Phase II clinical trial [12] which ended in 2011 with no better results than placebo.

In 2016, a Phase II clinical trial investigated the safety and efficacy of RT-001 as treatment for Primary Axillary Hyperhidrosis[13]. This molecule comprises the Botulinum toxin type A conjugated with two TAT moieties, and was formulated as a topic gel. The identical molecule was used in a Phase III clinical trial [14] for the treatment of moderate to severe lateral canthal lines. The trial was completed in September 2016, the data of this trial are not currently available.

All these clinical studies proved the safety of CPP-drugs but they show that additional developments of CPPs chemistry and efficacy could have a significant positive impact on human pharmacology in the next years.

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[12] Registered on ClinicalTrials.gov, Identifier: NCT 01135108

[13] Registered on ClinicalTrials.gov, Identifier: NCT 02655732

[14] Registered on ClinicalTrials.gov, Identifier: NCT 02580370

2. AIM OF THE STUDY

To date, Spinal Muscular Atrophy (SMA) is the first cause of infant mortality among neuromuscular disorders. It is caused by a homozygous mutation in the *SMN1* gene, which produces a non-functional SMN protein. From a clinical point of view, the affected patients are classified into 5 forms of SMA, from the most severe form (SMA 0) to the mildest one (SMA IV). This phenotypic heterogeneity is mainly due to the presence, in the human genome, of a paralogous gene, *SMN*2. A single nucleotide substitution in the *SMN*2 gene leads to the skipping of the exon 7 in the vast majority of the mRNA and the production of a truncated or unstable SMN protein for a 90%, and of a full-length SMN protein for a 10%. The severity of the pathology is inversely related to the number of *SMN*2 copies in the genome.

One possible therapeutic strategy for SMA is the modulation of the alternative splicing of the mRNA on the *SMN*2 gene, promoting the inclusion of the exon 7 and the expression of a full length functional SMN protein.

To date, the only approved therapy for SMA is the use of an Antisense Oligonucleotide (ASO), Nusinersen, which binds a specific region on *SMN*2, called ISS-N1, and block sterically the gathering of the splicing machinery that causes exon 7 skipping. ASOs with different chemistry have been already studied in animal models and in patients. In 2014, our group tested a particular ASO, called Morpholino, which targeted the 10-34 region of the ISS-N1 of the SMN2 intron 7 (MO-10-34). Our data showed that if MO-10-34 was administered at birth, the life expectancy and the neuromuscular function strikingly improved in the transgenic model of SMA [83].

It has been proved that the use of different sequences within and adjacent to ISS-N1 causes different therapeutic effects [83] and so the refinement of the target sequence could be a promising tool to obtain a better therapeutic effect. Additionally, a combined administration of multiple ASO oligomers has never been evaluated in detail. For all these reasons we decided to test new MO sequences using also a combined administration of them to improve the splice-correction effect of a single sequence.

One of the pitfalls of ASOs therapy is the need of a relative invasive administration into the cerebrospinal fluid (CSF) by lumbar puncture to reach the central nervous system (CNS). To solve this problem, different delivery strategies have been explored. One of these is the use of cell-penetrating peptides (CPPs), such as short peptide sequences of 5-40 amino acids that can pass through biological membranes. Due to their good efficacy and low toxicity *in vivo*, CPPs are often used as biological carriers. When they are linked to an ASO, they can improve its pharmacokinetic properties and bio-distribution, supporting the chance of their application in clinic.

We proposed to test if the conjugation of our validate MO sequence to different CPPs could increase the distribution and the delivery of MO in the CNS. We also compared two different administrations, the local and the systemic one, in a murine SMA model, in order to determine the most efficient CPP.

To summarize, the specific aims of this study are:

- the validation of new MO sequences alone or in combination in our *in vitro* and *in vivo* SMA models;
- the evaluation of the conjugates CPPs-MO in terms of:
    - ability to cross the Blood Brain Barrier (BBB) after systemic administration

- capacity to increase tissue distribution in brain and spinal cord
- opportunity of expanding the therapeutic window, allowing the treatment of SMA mice in a symptomatic phase.

This study will prove the possibility to use a CPP as a biological carrier for ASOs, allowing a systemic, non-invasive administration of the best conjugate. We expect to optimize our results in a preclinical context and to translate them in clinical trials and in clinical practice for the patients.

3. MATERIALS AND METHODS

3.1 PHOSPHORODIAMIDATE MORPHOLINO OLIGONUCLEOTIDES

The sequences of MO investigated in this study are designed to refine a 15 nucleotides inhibitory sequence downstream of the 5' splicing site in the intron 7 of the *SMN*2 gene. This element is identified as intronic splicing silencer N1 (ISS-N1) as previously described. Oligonucleotide nomenclature identifies the intronic annealing coordinates, thus MO (-10-34) refers to a molecule annealing between bases 10 and 34 from the beginning of *SMN*2 intron 7.

The list of tested MOs is here provided:

- MO-10-34:GTAAGATTCACTTTCATAATGCTGG
- MO-Scr: GTAACATTGACTTTGATATTCCTGG
- MO-A:TCATAATGCTGGCAGAC
- MO-B: GTAAGATTCACTTTCATAATGC
- MO-C: GATTCACTGTCAGAAGGCTGGCAGAC
- MO-D: GATTCACTCTCACAACGCTGGCAGAC

All MO oligomers were manufactured by Gene Tools (Philomath, USA) with a protocol previously reported [139] and have been designed taking into account analysis by bioinformatic tools to target ISSN-1.The previously validated MO-10-34[83] was used as reference sequence and the scr-MO was used as negative control sequence.

The novel MO oligomers (MO-A, B, C, D) were synthesized to match the region downstream of exon 7; in particular, MO-A and MO-B precisely matched the intronic ISSN-1 motif of the *SMN2* gene, while

only the first 10 nucleotides of MO-C and MO-D perfectly complementary bound to the ISSN1 intronic motif.
All the sequences were resuspended in sterile saline (0.9% sodium chloride) solution at the appropriate concentration for injection.

3.2 CONTROL CELL LINE

We used Hela cells as human immortalized wild-type line. The culture medium for this line was Dulbecco's modified Eagle's medium (MEM)/F12, with 15% Fetal Bovine Serum, 1% pen/strep, 1% amphotericin B (Life Technologies).1.5X 10^4cells were cultured into 24-well and subsequently maintained for 24 hours prior to transfection.

3.3 REPROGRAMMING OF HUMAN SOMATIC CELLS INTO IPSC

We reprogrammed SMA type 1 and wild-type skin fibroblasts into induced pluripotent stem cells (iPSCs) (n=3 per condition). The reprogramming has been performed using a non-integrating viral method, based on the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Life Technologies). The non-integrating Sendai virus (SeV) has been used to carry the 4 transcription factors described by Yamanaka at al. [150]: Oct4, Sox2, Klf4 and c-Myc. The reprogramming rate is guaranteed by the expression efficiency of these 4 transcriptional factors.
The first step of the reprogramming method consisted in culturing the SMA fibroblasts in DMEM high glucose (Life Technologies) supplemented with 15% fetal bovine serum (FBS) (Euroclone) and antibiotics until 50 to 80% confluence. The viral vectors (KOS, hc-Myc

and hKlf4) with the appropriate MOI (respectively: MOI=5-5-3) were added.

24h after the treatment, the medium was changed in order to eliminate the residual reprogramming vectors.

After 5 days, reprogrammed cells were detached and transferred in plates pre-coated with a layer of human embryonic fibroblasts (ATCC). At day 8, the medium was substituted with a specific medium for iPSCs (Essential 8 Medium, Life Technologies).

After 9 days, cells have been monitored for morphological changes towards iPSCs colonies, that usually were detected between two and four weeks after transduction. Then, colonies were isolated and grown in Essential 8 Medium. For the first passages, cells were grown on fibroblasts layer. Gradually, iPSCs colonies were transferred into plates pre-coated with a thin layer of cultrex (Cultrex® Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract Path Clear; Thema Ricerca), which is needed for their proper adhesion.

3.4 DIFFERENTIATION OF IPSCS INTO MOTOR NEURONS

We differentiated MNs applying a multistep differentiation method implemented for pluripotent stem cells [152]. To obtain MNs, we seeded iPSCs with neuronal medium [DMEM/F12 (Gibco, Invitrogen), with MEM nonessential amino acids, N2, and heparin (2 µg/mL, Sigma-Aldrich)]. To determine neural caudalization, we supplemented the medium with retinoic acid (RA) (0.1 µM, Sigma-Aldrich) after 10 days. At day 17, we collected the posteriorized neuroectodermal cells. These cells aggregates were resuspended for 1 week in the same medium with RA (0.1 µM) and sonic hedgehog (SHH) (100–200 ng/mL, R&D Systems Inc.). On day 24, we supplemented the medium with

additional growth and neuroprotective substances [e.g., brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and insulin-like growth factor-1 (IGF1) (10 ng/mL, Peprotech). To select MNs we used a gradient centrifugation protocol.

3.5 MORPHOLINO TRANSFECTION

To promote transfection efficiency, nucleofection was undertaken with the Neon Nucleofection System (Life Technologies) following the manufacturer guidelines. Briefly, cells Hela cells and SMA iPSCs were detached, counted and centrifuged. The cell pellet was resuspended in 100µl of Resuspension Buffer (Life Technologies).

Twenty micrograms MO were added to the cells and mixed by vortexing. Since MO-B and MO-D were the most efficient sequences, we employed a combination of these two oligomers, using the same total dose (10 µg MO-B + 10 µg MO-D).

Each sample was transferred with the Neon TM Pipette to a nucleofection cuvette and nucleofected using the proper set pulse condition. After nucleofection, the cells were then transferred on the culture plate previously prepared with pre-warmed culture medium. Transfected HeLa cells were collected 72h after treatment for immunocytochemical and western blot analyses. Treated SMA iPSCs and MNs were collected at different time frames: 72h, 7 days and 21 days after nucleofection.

To promote the transfection of MNs, we used the endo-porter kit (Gene Tools), following the manufacturer guidelines. Briefly, we started from plated MNs with a confluence of 60-80%. We replaced culture medium with fresh complete medium (containing up to 10% serum). We added a combination of two oligomers (10 µg MO-B + 10 µg MO-D) to the

medium and 6 µl of Endo-porter for every 1ml media and immediately swirl to mix (the final Endo-porter concentration should be of 6 µM).
The nucleofected MNs were collected 72h after the treatment for immunocytochemical analysis.

3.6 IMMUNOCYTOCHEMISTRY

3.6.1 IPSCs CHARACTERIZATION

Immunocytochemistry for pluripotency markers was performed on iPSCs. Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 10% BSA and 0.3% TritonX-100 in phosphate-buffered saline solution (PBS) for 1 hour at room temperature. Cells were exposed overnight at 4°C to primary antibodies (SOX2, OCT4, NANOG mouse monoclonal, Chemicon, 1:500; SSEA3, SSEA4, mouse monoclonal, Chemicon, 1:500, TRA1-60, TRA1-81 mouse monoclonal, Chemicon, 1:500; anti- SMN, 1:100, BD). The day after the cells were washed 3 times with PBS solution to remove the excess of unconjugated antibodies, and then incubated for 1 hour and 30 minutes at room temperature with secondary antibodies conjugated with the fluorophores Alexa Fluor 488 or 568 (anti-mouse antibody, 1:1000, Invitrogen) for pluripotency markers or with the secondary anti- mouse FITC-conjugated antibody (1:100, Dako). Nuclei were stained with DAPI. Images were acquired using the LEICA LCS2 confocal microscope. Results are expressed as mean ± s.e.m. for five independent experiments.

3.6.2 GEMS QUANTIFICATION

The SMN expression level was determined in Hela cells and in SMA iPSCs also evaluating by immunocytochemistry the presence of aggregates of SMN called “gems”. The cells were grown on slides were washed with PBS and fixed with 1:1 acetone:methanol for 5 min. Subsequently, they were incubated with a solution of 10%BSA, 2%Triton in PBS. Then, after washing cells were incubated with an antibody against SMN in 3% BSA overnight at 4 degrees (1:100, BD). Cells were washed with PBS and exposed for 1 hour to secondary antibodies (Alexa Fluor, Invitrogen). After washes with PBS, the cells were then incubated with DAPI (Invitrogen).

The number of gems was quantified following the protocol reported in a previous study [151]. Cells were examined with an SP2 AOBS confocal microscope. Two independent investigators examined at least 100 cells in randomly selected fields from each slide and recorded the gems number and the percent of cells with gem-positive nuclei in the different treatments.

3.6.3 MOTOR NEURON PHENOTYPIC ANALYSIS

We evaluated the MN phenotype by morphological and immunohistochemical analysis. We used the following antibodies: TuJ1 (1:200, Millipore), OLIG2, (1:500, Santa Cruz), ISLET1 (1:200, Millipore), HB9 (1:200 Millipore), ChAT (1:200, Millipore), MAP2 (1:100; Sigma), and SMI32 (Covance, 1:500). The method is described in detailed above for iPSC staining. For phenotypic analysis, the percentage of positivity in a sample was obtained by averaging proportions of a specific cell type in each of 10 randomly chosen fields. Five independent experiments in triplicate were performed. Morphometric and MN count analysis were carried out as previously

described by us. Results are expressed as mean ± s.e.m. for five independent experiments.

3.7 CONJUGATION OF MOs TO CELL PENETRATING PEPTIDES

The peptides we chose to be conjugated with MO were the following (**Figure 9**):

- HIV1 TAT peptide (sequence: YGRKKRRQRRRQ),
- two polyarginine peptides (namely R6 and r6, sequence RRRRRR). The two peptides are made up by six arginine with a L (R6) or D (r6) enantiomeric configuration.
- (RXRRBR)2 XB peptide (namely RXR).

All the peptides were synthetized by the commercial peptide supplier Peptide 2.0. All CPPs were conjugated to the previously validated MO sequence (MO-10-34) [83], synthetized by Gene Tools, LLC. Each peptide have been linked to the 5' end of the MO through non-cleavable maleimide bond as reported [153]. The conjugation process was performed by our external collaborator Hong Moulton (University of Oregon) using a method already described [154, 155]. The lyophilized compounds were dissolved in sterile saline solution to obtain the final correct concentration.

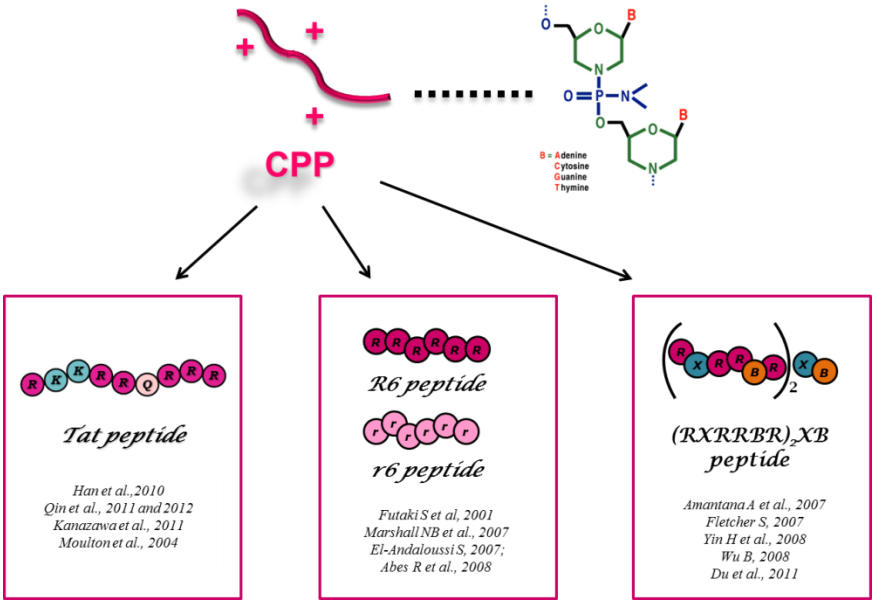

Figure 9: Cell Penetrating Peptides tested in this study

3.8 SMA TRANSGENIC MICE

All the transgenic animals were purchased from The Jackson Laboratory (**Figure 10**). All animal experiments were approved by the University of Milan and Italian Ministry of Health review boards, in compliance with US National Institutes of Health Guidelines [53].

We employed the transgenic mouse model usually identified as SMAΔ7 (*SMN2+/+; Smn-/−; SMNΔ7+/+*). This triple mutant mouse harbors two transgenic alleles and a single targeted mutant. The Tg(SMN2*delta7)4299Ahmb allele is a SMA cDNA lacking exon 7 while the Tg(SMN2)89Ahmb allele is the entire human *SMN2* gene.

Animals that are homozygous for the knockout *Smn* allele and homozygous for the two transgenic alleles show signs/symptoms and neuropathology that mimic SMA type 1. At birth, triple mutants are smaller than wild-type littermates. By P5, signs of muscle weakness

are evident and become progressively more severe over the following week. The mice cannot stand or walk on their limbs. Mean survival is approximately 13 days.

Figure 10: Genetically engineered and mutant mice from the Jackson Laboratory: gene and allele information (http://jaxmice.jax.org/strain/007952.html).

Non affected heterozygous male and female (*SMN2+/+; Smn+/−; SMNΔ7+/+*) were bred and pups were identified by genotyping as described below.

3.9 MOUSE GENOTYPING

2 mm of tail tissue from SMA pups was cut and collected into an Eppendorf tube.

For the DNA extraction, 100 µL of 50 mM NaOH were additioned to each sample and each tube was located in the water bath at 95ºC for

10 minutes. 25 µL of 1M TrisHCl pH 8.0 solution were then added to each sample. Then, samples were mixed by vortexing. After the DNA extraction, it was amplified carrying out a standard PCR with 35 cycles. The conditions and the primers sequences are here presented:

• Primer sequences: **RIMER**	**PRIMER TYPE**	**SEQUENCE 5' TO 3'**
oIMR3679	Transgene	TCCATTTCCTTCTGGACCAC
oIMR3680	Transgene	ACCCATTCCACTTCCTTTTT
oIMR8744	Internal Positive Control Forward	CAAATGTTGCTTGTCTGGTG
oIMR8745	Internal Positive Control Reverse	GTCAGTCGAGTGCACAGTTT

- PCR: components and cycling

REACTION COMPONENT	**VOLUME (µL)**	**FINAL CONCENTRATION**
ddH_2O	3.56	-
10X AB PCR BufferII	1.20	1-00 x
25 mM $MgCl_2$	0.96	2.00mM
2.5 mM dNTP	0.96	0.20mM
20 µM oIMR3679	0.60	1.00 µM
20 µM oIMR3680	0.60	1.00 µM
20 µM oIMR8744	0.20	0.33 µM
20 µM oIMR8745	0.20	0.33 µM
5 Mm DNA loading Dye	1.66	0.69 µM
5 U/µL Taq DNA Polymerase	0.06	0.03 U/µL
DNA sample	1.50	-

- Separation of PCR products by gel electrophoresis on a 1.5% agarose gel.

3.10 MORPHOLINO ADMINISTRATION IN SMA MICE

The most efficient MOs *in vitro* were tested *in vivo* in heterozygous SMA mice (genotype: *Smn+/-, hSMN2+/+, SMNΔ7+/+*) to confirm the increase of SMN protein levels (**Table 3**).

The MOs tested were MO-B, MO-D and MO-B + MO-D. At post-natal day 1 (P1), the different MO solutions were administered intracerobroventricularly (ICV) and subcutaneously (SC)at a dosage of 12 nmoles for each injection in heterozygous pups. At P3 extra 12 nmoles were injected SC. The same dose was used for the combined delivery of MO-B + MO-D, 6 nmoles MO-B plus 6 nmoles MO-D for each injection.

The volume of each injection was 2.4µL. Treated mice were sacrificed at P7 and brain and spinal cord were collected for further neuropathological and proteomic analyses.

MOs were suspended in sterile saline, and mixed with Evans Blue (final concentration 0.04%) and stored at −20°C.

The ICV injection was performed as previously described [83] cryo-anesthetized mice were hand-mounted over a back-light to visualize the intersection of the coronal and sagittal cranial sutures (bregma).A fine-drawn capillary needle with injection assembly was then inserted 1 mm lateral and 1 mm posterior to bregma and then tunnelled approximately 1 mm deep from the skin edge, corresponding to the ipsilateral lateral ventricle. An opaque tracer (Evans Blue, final concentration 0.04%) was mixed to the reagent so that the borders of the lateral ventricle could be visualized after the administration of the MOs.SC injections were performed as previously described by Hua team [74].

Treatment group	n	Mice genotype	Treatment	Dosage	Route and time of administration
Control group	3	Hetero	SCr-MO	12 nmol/g	ICV + SC at P1 and SC at P3
1	3	Hetero	MO B	12 nmol/g	ICV + SC at P1 and SC at P3
2	3	Hetero	MO D	12 nmol/g	ICV + SC at P1 and SC at P3
3	3	Hetero	MO B +MO D	6 nmol/g MO B+6 noml/g MO D	ICV + SC at P1 and SC at P3
4	3	Hetero	MO 10-34	12 nmol/g	ICV + SC at P1 and SC at P3

Table 3: Description of treatment groups. Treatment dose is referred as dose/ injection. Abbreviations: scr, scrambled; nmol, Nano Moles; ICV, intracerebroventricular injection; SC, subcutaneous injection; P, post-natalday; Heterozygous mice genotype: *Smn+/-,SMN2+/+, SMNΔ7+/+.*

3.11 DELIVERY OF CPP-MO COMPOUNDS

Different groups of animals were treated with CPP-MO conjugates.

First, we treated affected symptomatic homozygous mice with TAT-MO in comparison to unconjugated MO-10-34. In particular, the first group of homozygous mice (n=9) received a low dose of TAT-MO (6 nmoles/g) ICV plus a 6nmoles/g SC at P5 and an extra 6nmoles/g TAT-MO SC at P7.A second group (n=14) of homozygous mice received a high dose of TA-MO (12nmol/g per injection) ICV plus SC at P5 and an extra SC injection at P7.The two groups of treated mice were compared to two control homozygous groups treated with unconjugated MO 10-34 at low dose (6 nmoles/g, n=10 treated mice) and high dose (12

nmoles/g, n=9 treated mice) following the same administration scheme (**Table 4**).

ICV and SC injection are already described in the previous section (3.10). Survival of the treated mice was monitored.

Treatment Group	n	Treatment	dosage	Administration route and time of treatment	Mean survival
High dose group	14	TAT-MO-10-34	12 nmol/g	ICV+SC at P5 plus SC at P7	47,14 d
Low dose group	9	TAT-MO-10-34	6 nmol/g	ICV+SC at P5 plus SC at P7	27,777 d
Control group	10	Unconjugated MO-10-34	6 nmol/g	ICV+SC at P5 plus SC at P7	25,2 d
Control group	9	Unconjugated MO-10-34	12 nmol/g	ICV+SC at P5 plus SC at P7	41 d

Table 4: Comparison between homozygous mice treated with TAT-MO and MO-10-34. Dose is referred as dose/injection. Abbreviations: d, days; nmol, NanoMoles; ICV, intracerebroventricular injection; SC, subcutaneous delivery.

In parallel, small pilot groups of non-affected heterozygous animals (Smn+/-, SMN2 +/+, SMNΔ7+/+) were injected with MO-(10-34) linked with one of the four different CPPs: TAT, R6, r6, RXR (**Table 5**). In this case, we used two routes of administration. We injected ICV plus SC with 2.4 µL/g of CPP-MO per injection, which corresponds to 12nmoles/g, at P1 and an extra dose SC at P3. Other pilot groups were treated intravenously (IV) into the facial vein with 12 nmoles/g of each CPP-MOs, which were further diluted to a final volume of 50 µL. The

same two protocols were applied for the delivery of unconjugated MO-(10-34) as positive control [156].

Treated mice were sacrificed at P7. The brain and the spinal cord were collected for further neuropathological and proteomic analyses.

Treatment Group	n	Treatment	dosage	Administration route and time of treatment	Mean survival
TAT group 1	1	TAT-MO-10-34	12 nmol/g	ICV+SC at P1 plus SC at P3	Sacrificed at P7
R6 group 1	1	R6-MO-10-34	12 nmol/g	ICV+SC at P1 plus SC at P3	Sacrificed at P7
r6 group 1	1	r6-MO-10-34	12 nmol/g	ICV+SC at P1 plus SC at P3	Sacrificed at P7
RXR group 1	1	(RXRRBR)2XB-MO-10-34	12 nmol/g	ICV+SC at P1 plus SC at P3	Sacrificed at P7
TAT group 2	1	TAT-MO-10-34	12 nmol/g	IV at P1	Sacrificed at P7
R6 group 2	1	R6-MO-10-34	12 nmol/g	IV at P1	Sacrificed at P7
r6 group 2	1	r6-MO-10-34	12 nmol/g	IV at P1	Sacrificed at P7
RXR group 2	1	(RXRRBR)2XB-MO-10-34	12 nmol/g	IV at P1	Sacrificed at P7

Table 5: CPPs-MO treatments in heterozygous mice. Dose is referred as dose/injection. Abbreviations: d, days; nmol, NanoMoles; ICV, intracerebroventricular; injection; SC, subcutaneous injection; IV, intravenous injection. Homozygous genotype: *Smn-/-, SMN2+/+, SMNΔ7+/+*; Heterozygous genotype: *Smn+/-, SMN2+/+, SMNΔ7+/+*.

The experiment for the evaluation of the efficacy of the conjugates on survival, neuromuscular function and neuropathological phenotype on symptomatic affected SMA mice are ongoing.

3.12 WESTERN BLOT ASSAY

Cells were washed with PBS solution and detached by mechanical scraping. Lysis buffer additioned with protease and phosphatases inhibitors mix (Pierce) was then added and the cells were lysed by sonication for 10 s on ice to avoid overheating. The lysates were then centrifuged at 13,500 rpm for 10 min at 4°C.

Twenty milligrams of frozen tissue were homogenized in 0.4 mL (brain) or 0.2 mL (spinal cord) of 1× protein sample buffer containing 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM Tris hydrochloride (pH 6.8), and 0.1 M dithiothreitol. Protein concentration was determined by Pierce Comassie Plus Protein Assay (an improved Bradford assay, Pierce).

Twenty micrograms of proteins were separated by electrophoresis using 12% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. Membranes were incubated with 1%BSA, 10% horse serum, 0,075% Tween 20 in TBS (20 mM Tris-HCl, 0.5 M NaCl) solution for 1 hour at room temperature (RT) to prevent any non-specific binding of antibodies. The membrane was then exposed overnight at 4°C to the primary antibody resuspended in saturating solution: anti-human SMN (1:15,000, amino acids 14–174, BD Biosciences) or anti-β-actin (1:250, Sigma) as a reference loading protein. After three washings of 15 minutes with TBS-T solution (20 mM Tris- HCl, 0.5 M NaCl, 0.05% Tween 20), the membranes were probed with secondary peroxidase-conjugated antibody (Invitrogen)

resuspended in saturating solution. After three washing with TBS-T, immune complexes were detected using chemiluminescent detection system (ECL solution, Amersham). Densitometric analysis was performed using Image J software.

3.13 BEHAVIORAL TESTS

The experiment for the evaluation of the efficacy of the conjugates on survival, neuromuscular function and neuropathological phenotype on symptomatic affected SMA mice are ongoing. All treated mice were monitored every day for morbidity, mortality and changes in weight by investigators that were blind to the treatment. Body weight was measured daily using a standard small animal balance. The mice were sacrificed at the clinical end point when they presented difficulties in feeding, a clear downward trend (mice with 30% weight loss and severe paralysis), and breathing problems.

Behavioral tests were carried out as previously reported [53, 74]. In particular, the neuromuscular function of treated mice was evaluated by the rotarod test, righting reflex assay and hind-limb suspension test (also referred as Tube Test).

The rotarod test comprises 4 phases of acceleration (4–40 rpm, maximum time 5'; Rota-Rod 7650; Ugo Basile). The length of time that each mouse remained on the rotarod the portion of no-fall tests were recorded.

Hind-limb suspension and righting reflex tests were performed as previously described [54, 157].

The righting reflex is employed to determine motor function of mice at early developmental ages. It defines the capacity of the mouse to perform the necessary movement to roll over from its back onto its

paws. Incapacity to right, or significant delays in righting point out defects in motor functioning. The test was assessed starting from P2.

For this test, each mouse was placed on its back on a flat surface in a supine position, Its success or failure to stably reposition on all four paws on the ground was assessed over a 30 s period (cut off time of 60 s)[60].

The hind-limb suspension test (HLST, a.k.a. the tube test) is designed for the evaluation of proximal hind limb muscles, weakness and fatigue in early stage of mouse development. It assesses also general neuromuscular function. The test is generally performed in 2 consecutive trials.

The mouse is placed head-down and hanging by its hind limbs from the edge of a standard 50 ml plastic centrifuge tube with a cotton ball cushion at the bottom to protect the animal's head upon its fall. The HLST results are expressed by the latency time to fall from the edge of the plastic tube and by the Hind-limb Score (HLS).

The HLS evaluates the positioning of the legs and tail. Based on the posture of mice. These scores are assigned following the criteria presented in a previous study [158]:

• score 4: indicates normal hind-limb separation with tail raised;

• score 3: weakness is evident and hind limbs are closer together but they seldom touch each other;

• score 2: hind limbs are close to each other and often touching;

• score 1: weakness is evident and hind limbs are almost always in a clasp position with the tail raised;

• score of 0: indicates constant clasping of the hind limbs with the tail lowered.

HLS scores are determined during the first 10 to 15 s of hanging from the plastic tube. In case the pups failed to hold onto the tube, an HLS score of 1 was given.

3.14 STATISTICAL ANALYSIS

We used Prism software for statistical analyses. All quantification data from immunocytochemical analyses and cell survival were expressed as mean values ± S.D or s.e.m. Differences between two means were analyzed using Student's t-test (two-tailed), and differences among more than two means were evaluated by one- or two-way ANOVA. When ANOVA showed significant differences, pair-wise comparisons between means was carried out using Tukey's post-hoc test. For the survival curve analysis, data are expressed as means plus standard errors. Kaplan–Meier curves were obtained from the survival data and evaluated by the Mantel–Cox log-rank test. In all tests, the null hypothesis was rejected when p was less than 0.05.

4. RESULTS

4.1 SMN UPREGULATION IN HUMAN WILD-TYPE CELLS USING NOVEL MO SEQUENCES

Our group have already validated a 25 nucleotides MO sequence to targeting the intronic splicing motif referred as ISS-N1 in the human *SMN2* gene [83]. This MO oligomer was identified as HSMN2Ex7D(10,34), or MO-(10-34), due to its ability to pair the exon 7 donor site.

Our group demonstrated the efficacy of this sequence in promoting exon 7 inclusion in the *SMN2* mRNA and in upregulating the levels of the functional full length isoform of the SMN protein both *in vitro* and *in vivo* [83].

We decided to compare the already tested efficacy of MO-(10-34) oligomer with four newly designed MO sequences (MO A, B, C, D), all tailored to bind the ISS-N1 region downstream of *SMN2* exon 7. In particular, while MO-A and B oligomers precisely overlap the target intronic splicing site of the SMN 2 gene, MO-C and MO-D sequences match only for the first 10 nucleotides the ISS-N1 splicing site.

MO novel sequences were produced by Gene Tools by an already described method of synthesis [159]. As negative control we used a scrambled MO sequence (scr-MO) that was designed using bioinformatics software tool to predict the most appropriate control sequence (Gene Tools, www.genetools.com).

The novel MO sequences and their positive/negative controls were tested in a human immortalized wild-type cell line, Hela cells. Cells were nucleofected with 20µg of the four MO oligomers or the scr-MO control sequence following the protocol described in manufacturer instructions.

Cells were collected72h after MO nucleofection for western blot and immunocytochemical to measure SMN levels.

Data obtained from western blot proved that all the four sequences are able to increase in the SMN protein levels compared to the scramble treated cells, and in particular MO-B and MO-D presented the highest activity among the newly designed oligomers (**Figure 11**). For this reason, we decided to combine the administration of MO B and MO D in order to verify the possible additive effect of the two compounds. The protein analysis revealed that the combination of these two MOs produced a greater effect than the two oligomers alone and it was similar to that obtained with MO-10-34 that serves as positive control ($p < 0.05$, **Figure 11**).

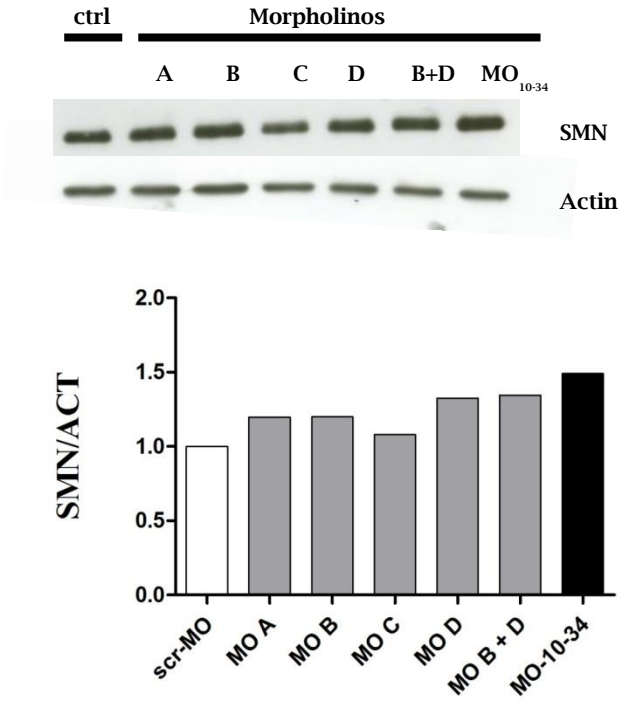

Figure 11: Western blot (upper panel) and densitometric analysis (lower panel) of wild-type HeLa cells treated with different MO sequences and scr-MO as negative control.

Another evaluated parameter was the presence of nuclear complexes, named gems. These structures are detectable only when SMN protein is present and are critical for the correct processing of RNA messengers. The gems number was quantified and expressed as the mean value of the ratio between the number of gems counted and the number of nuclei present in each examined area.

The immunocytochemical analysis (**Figure 12**) revealed that MO-B, D, B+D and MO-(10-34) were able to significantly up-regulate the number of gems in particular the gems/nuclei ratio respect to scr-MO treated controls ($p<0.05$, **Figure 12**). No significant upregulation was detected in the presence of MO-A or MO-C. Moreover, the most significant up-regulation in the gems and in the gems/nuclei ratio was determined after the co-administration of MO-B + MO-D (mean ratio = 0.71), as already shown by western blot analysis. These results were statistically significant as demonstrated by one-way ANOVA and post-hoc Tukey's test ($p=0.0048$) (**Figure 13**).

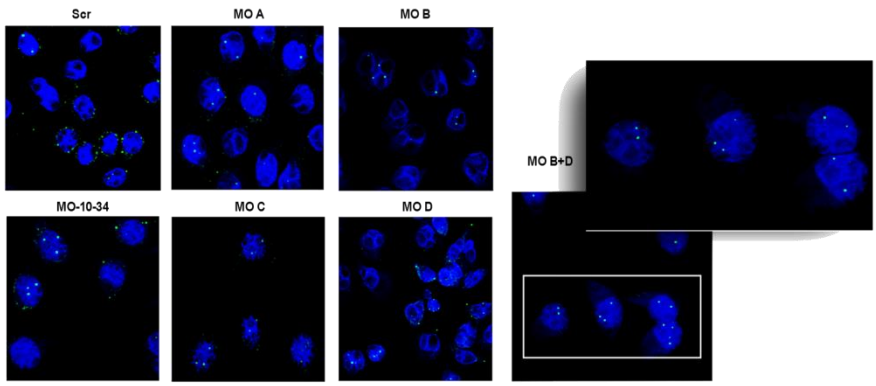

Figure 12: Immunocytochemical analysis of treated wild-type HeLa cells with MOs. Nuclear gems (green) were observed in the nucleus of all treated cells and the greatest increase in their number was detected after the co-treatment with MO-B + MO-D. Nuclei are stained with DAPI

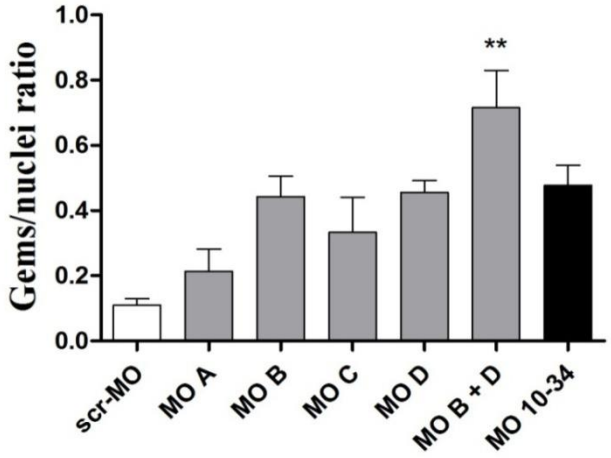

Figure 13: Analysis of the means of gems/nuclei ratio in wild-type HeLa cells after exposure with different MO sequences. The greatest increase was observed after the co-treatment with MO B + MO D

4.2 MO NOVEL SEQUENCES UP-REGULATE SMN PROTEIN IN SMA iPSCs

To obtain a suitable SMA *in vitro* model, we reprogrammed patients (SMA type 1) and wild-type skin fibroblasts in iPSCs (n = 3 per condition). For this purpose, we used a non-integrating viral approach based on the use of Sendai Virus, to efficiently transfer the required stem transcription factors (OCT4, SOX2, KLF4 and c_Myc, OSKM) described by Yamanaka et al.[150]. After the reprogramming iPSCs colonies with typical ES-like morphology appeared in culture and were manually isolated. The cells showed the characteristics nuclear/cytoplasm ratio and are pluripotent since they can originate

embryoid bodies with the three germ layers. The iPSC correctly expressed the pluripotency markers SOX2, OCT4, NANOG, SSEA3, 4 and TRA1-60 and TRA1-81 confirming the successful reprogramming (**Figure 14**). Karyotypes were normal and the correct identity of the cells were confirmed by DNA fingerprinting.

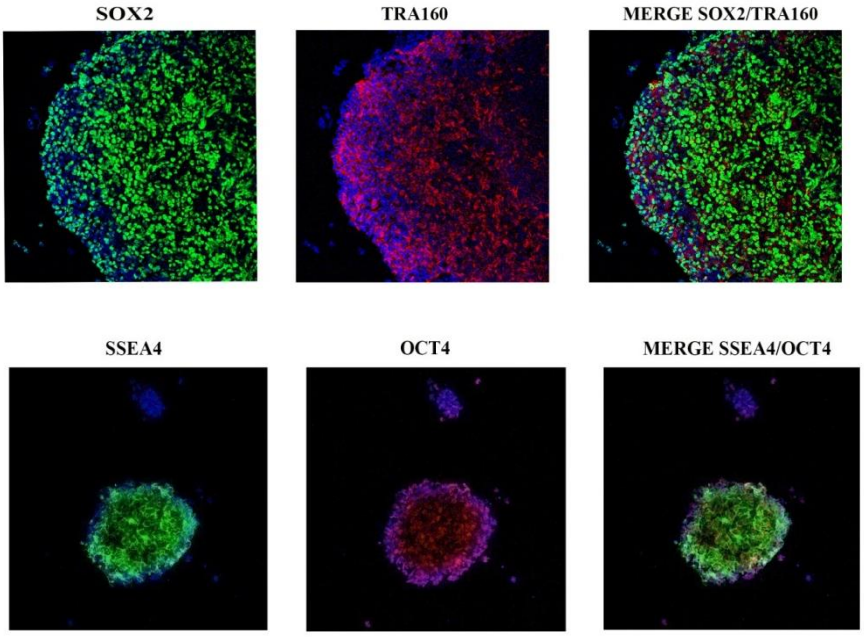

Figure 14: Immunocytochemical analysis of the expression of pluripotency markers in human SMA iPSCs. iPSCs colonies stained positive for the pluripotency markers SOX2, TRA160, SSEA4 and OCT4, confirming the acquisition of a proper phenotype after viral reprogramming

Once the line was stabilized, SMA iPSCs were treated with n with the different novel MO oligomers (20ug) by nucleofection following the same method employed for the HeLa cells. After treatment, iPSC cells were collected at different time points: 72h, 7 days and 21 days order to test the time/efficacy curve.

We performed a proteomic analysis with western blot. MO-B and D were the two most effective oligomers in up-regulating the SMN protein level among the four compounds and, also in this case, the co-administration of MO-B + MO-D was more effective than the treatment of the single oligomers at the same dose 72h after nucleofection (**Figure 15**).

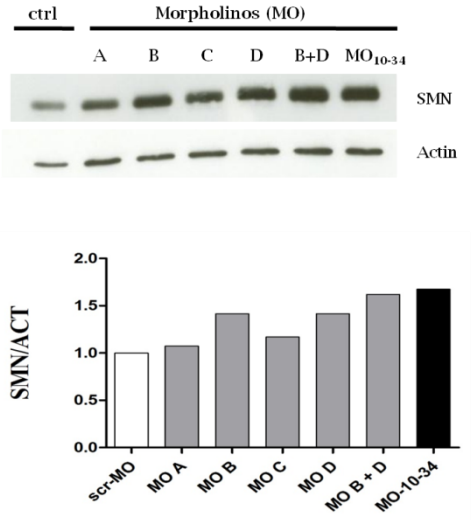

Figure 15: Western blot (upper figure) and densitometric analysis (bottom figure) of SMA iPSCs treated with different MO sequences and harvested 72h after treatment showed that the co-administration of MO-B + MO-D was more efficient than other MO sequences in up-regulating SMN level and it was similar to MO-10-34

Immunocytochemical analysis of iPSCs harvested 72h after treatment (**Figure 16**) demonstrated that the gems/nuclei ratio was higher in the cells treated with MO-B + MO-D (mean ratio =0.55) and was proved to be statistically significant by one-way ANOVA and post-hoc Tukey's test (p=0.0011), confirming the result obtained in wild-type cells (**Figure 17**).

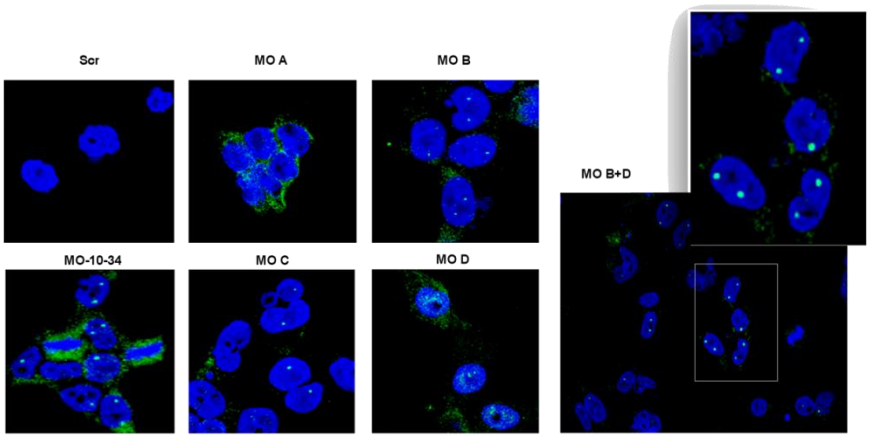

Figure 16: Immunocytochemical analysis of treated SMA iPSCs. Nuclear gems (green) were detectable in all treated cells and the greatest increase in the number was seen after the co-treatment with MO B + MO D. Nuclei are labeled with DAPI

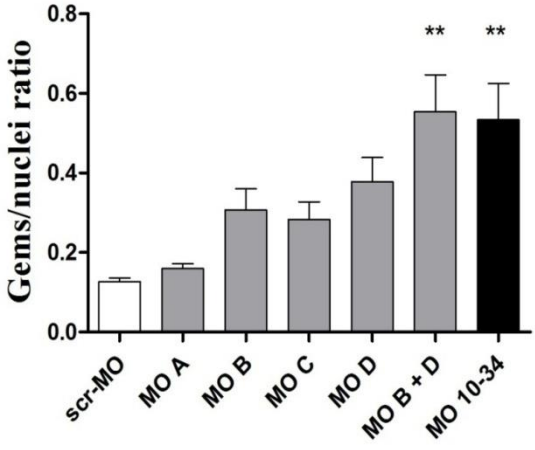

Figure 17: Comparison of the means of gems/nuclei ratio in human SMA iPSCs treated with different MO sequences. The greatest increase was seen after the co-treatment with MO B + MO D

In order to assess whether the splicing correction effect was maintained after multiple replication cycles, we collected treated iPSC cells at different time of harvesting after nucleofection. The co-treatment with MO-B + MO-D oligomers allows to obtain higher level of SMN full-length for a longer time period, as shown by western blot analysis of iPSCs collected after21 days from MO-nucleofection (**Figure 18**).

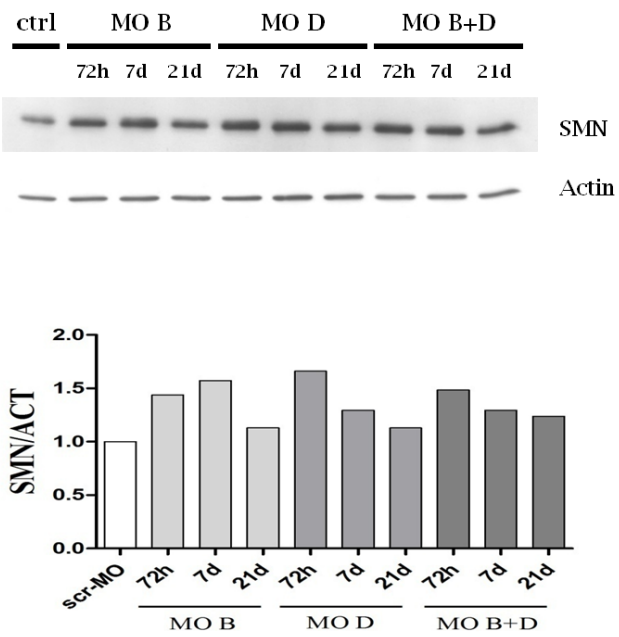

Figure 18: Western blot (upper figure) and densitometric analysis (bottom figure) of SMA iPSCs nucleofected with MO-B, MO-D or MO-B + MO-D and collected 72hours, 7days and 21 days after treatment showed that the co-treatment with MO B + MO D maintained higher levels of SMN for a longer times frame.

These results suggest that the combined administration of different MO sequences can act in a synergistic increasing their efficacy. Remarkably, the enhanced efficacy is clearly detectable evident despite

the total MO dose is equal to that used in the single sequence treatment. This finding is important in the design of future experimental therapeutic protocol with ASOs.

4.3 TREATMENT OF IPSC- DERIVED SPINAL MOTOR NEURONS WITH MO-B+D AMELIORATE THE SMA DISEASE PHENOTYPE

We analyzed whether MO-B+D treatment can up-regulate SMN level also in differentiated SMA iPSC-MNs ameliorating their phenotype. Differentiation of spinal MNs from all iPSC lines was obtained by a multistep differentiation method previously implemented for human iPSCs based on the exposure to RA and SHH. After 4–5 weeks, MNs were obtained that were positive for MN-specific markers including spinal cord progenitor antigens (HB9, ISLET1, and OLIG2) and pan-neuronal protein (TuJ1, Neurofilament, and MAP2). The majority of these HB9/ISLET1-positive cells stained for Choline Acetyl Transferase (ChAT) and expressed the MN marker SMI-32, confirming the acquisition of a MN phenotype. At 10 weeks, we detected a decrease in the number of mature MNs in the untreated SMA-iPSC derived MNs respect to wt-iPSCs ($P < 0.01$, **Figure 19**). SMA-iPSC MNs that were exposed to MO-B+D exhibited increased survival and neurite length respect to MNs differentiated from SMA-iPSCs-MNs treated with control vector ($P < 0.01$, ANOVA). These data support the fact that SMA disease hallmarks in human SMA-MN caused can be rescued by *SMN2* gene modulation using MO-B+D.

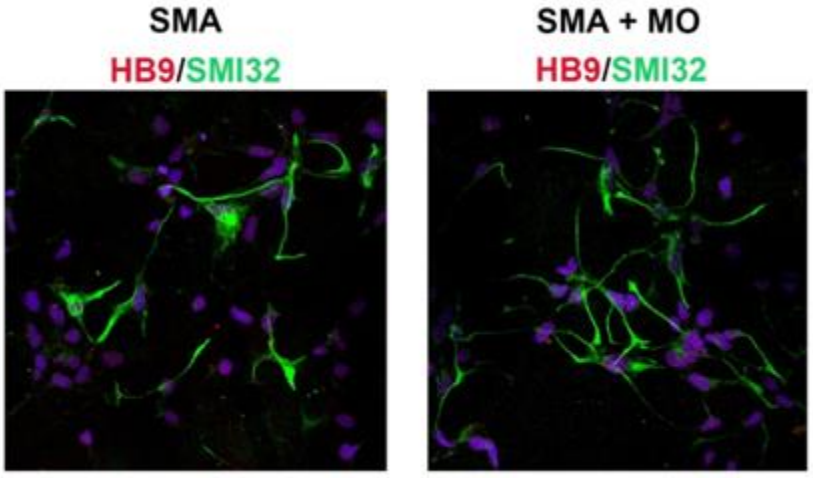

Figure 19: MO-B+D ameliorates the SMA phenotype in iPSC-MNs, increasing cell survival and neurite length in long-term cultures.

4.4 NOVEL MO SEQUENCES ARE ABLE TO INCREASE SMN PROTEIN LEVELS IN SMAΔ7 MICE

The MO oligomers that resulted more efficacious in increasing SMN level in vitro models, were then tested in vivo in the SMAΔ7 transegnic mouse model. Non affected heterozygous mice were injected with different sequences (MO-B, MO-D, MO-B + MO D or MO-(10-34)) along the therapeutic protocol design presented in the Materials and Methods section. A subgroup of animals was administered with the negative control sequence scr-MO. Injected animals were sacrificed at P7 and brains and spinal cords were collected for proteomic studies.

Western blot analysis revealed increased level of SMN full-length in all MO-treated tissues as compared to negative controls. However, MO-B and MO-D in single administration failed to significantly increase SMN protein level. Instead, the combination of MO-B + MO D sequences

was able to significantly increase the SMN protein in the CNS of treated animals. It has to be notice that this combination was slightly less effective than MO-10-34 in the mouse brain (**Figure 20 A** and **B**), but more efficacious in the spinal cord (**Figure 20 C** and **D**).

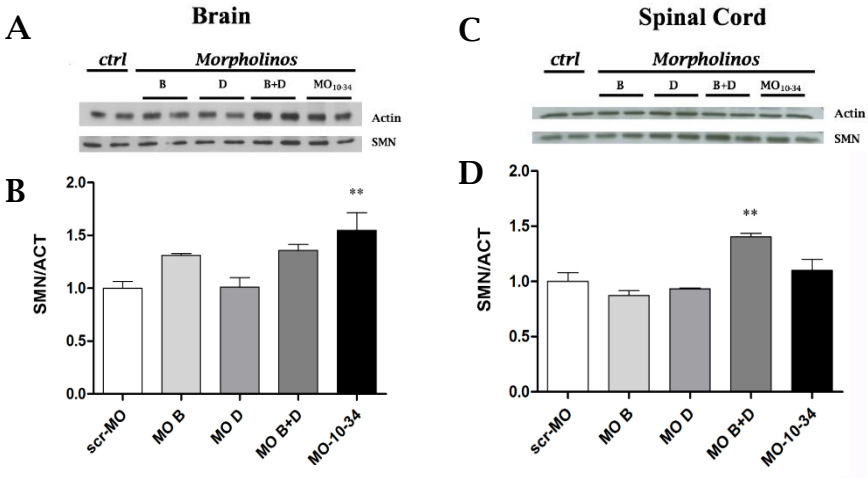

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Figure 20: Western blot (**A**) and densitometric analysis (**B**) of brain samples of SMA mice treated with the different MO sequences showed that the efficacy of co-administration of MO B + MO D was similar to that obtained with MO-10-34. Western blot (**C**) and densitometric analysis (**D**) of brain samples of SMA mice treated with the different MO sequences showed that the of co-administration of MO-B + MO-D was more efficient than other sequences and MO-10-34 in up-regulating SMN.

Our experiments confirmed that the inclusion of the exon 7 in the SMN2 transcript is a suitable and efficient strategy to increase the SMN protein levels. Furthermore, the use of combined alternative MO sequences can improve the effect of SMN2 splicing modulation and have a powerful impact on SMN protein levels.

4.5 THE TREATMENT WITH THE TAT-MO IMPROVED THE SURVIVAL OF SMA MICE

Despite being used in a wide range of human diseases as a promising strategy for their treatment, antisense oligonucleotides (ASOs) have a low cellular uptake and limited bioavailability. These issues can be overcome by conjugation with cell-penetrating peptides (CPPs), due to their ability to translocate across biological membranes, but this approach has never been explored in SMA.

One of the first CPP that has been reported in the literature [153, 160-162], and one of the most investigated in experimental settings, is the TAT peptide, so we choose it to explore the possibility and efficacy of the MO conjugation with CPP strategy for the improvement of ASO therapeutics.

Our goal was to demonstrate that TAT-MO was able to cross the BBB in SMA treated mice, improving their survival. Usually, the mean survival of the untreated mice is 13 days, with the manifestation of the first symptoms of the disease at post-natal day 4 (P4). So we decided to treat the mice at post-natal day 5 (P5).

Since in the past years we previously proved the efficacy of MO-(10-34) oligomers, we decided to link TAT peptide to this sequence.

First, we injected TAT-MO compound directly into the central nervous system (CNS) of symptomatic SMAΔ7 mice by intracerebroventricular (ICV) administration at P5, in combination with a subcutaneous (SC) administration at P5 and P7 (**Table 4**, Materials and Methods).

We tested two different doses of TAT-MO: one subgroup of symptomatic SMA animals was injected with 6nmoles/each injection of the conjugate and the results obtained were compared to the ones obtained from a second subgroup injected with unconjugated MO, as control. Data analysis demonstrated a relatively modest increase in

survival (mean survival 27.8 days) of the TAT-MO injected mice, respect to the subgroup injected with unconjugated MO (mean survival 25.2 days), even of the results do not reached significance.

The other tested dose was 12 nmoles per each injection (same administration protocol), in order to observe a dose-dependent increase of survival. The maximum survival of the group treated with a higher dose was 104 days (mean survival: 47.14 days, **Figure 21**) compared to the unconjugated MO (mean survival: 41 days, **Figure 21**). We observed that the 5 mice treated with TAT-MO (12 nmoles) survived more than 50 days compared to 2 mice treated with unconjugated-MO (12 nmloes).

Our data were statistically significant ($\chi 2 = 10.08$; $p=0.0015$). These results support that the effects of the TAT-MO is dose-dependent.

In general, we demonstrated that TAT-CPP had a promising safety/efficacy profile and, if conjugate to MO-10-34, was able to increase the survival of treated mice, enhancing the cellular uptake of MO oligonucleotides.

These results represent the ground for the design of further experiments to select and validate new and more efficacious CPPs to improve the cellular entrance of ASO and their tissutal distribution, in particular in the CNS, even in symptomatic phase.

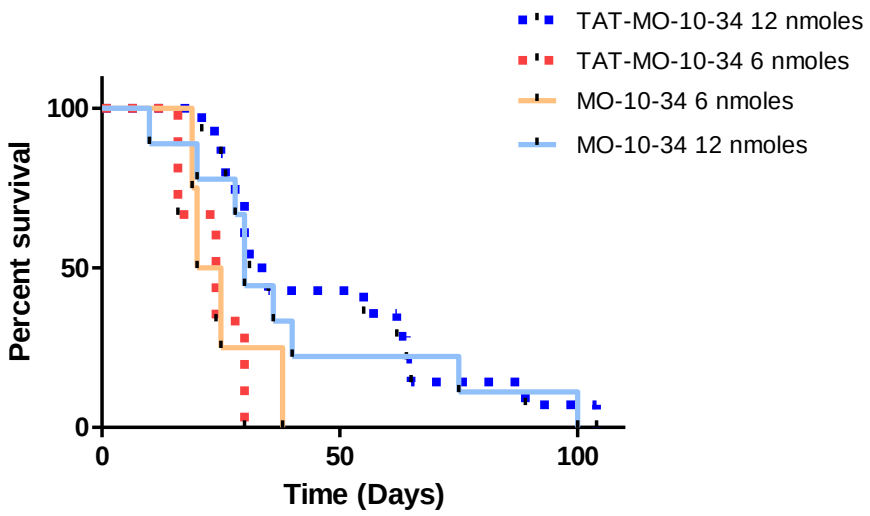

Figure 21: Survival curve of severe SMA mice treated with unconjugated MO or with TAT-MO by intrathecal injection. Mice treated with 6nmoles of TAT-MO showed a modest increase in survival compared to unconjugated MO. Mice treated with 12nmoles of TAT-MO showed a significant increase in survival compared to unconjugated MO.

4.6 CPPs-MO EFFICIENTLY INCREASED SMN AFTER LOCAL ADMINISTRATIONIN SMA Δ7 ANIMALS

The encouraging findings obtained after the TAT-MO injection in SMA Δ7 animals led us to test the capacity of other novel CPPs to promote the MO cell-entrance and the efficacy of MO-(10-34). We assessed the ability of different CPP-MO compounds to promote the correct inclusion of exon 7 in the *SMN2* transcript *in vivo*. The final goal was to identify the best CPP-MO compound to test it further in a wider subset of SMA animals to obtain efficacy data.

We considered four different CPPs: TAT, two polyarginine peptides (calledR6 and r6) and the (RXRRBR)2-XB peptides, taking into account their efficiency and safety described in literature.

A group of non-affected heterozygous SMA-Δ7 animals was injected with unconjugated MO-10-34 or with scr-MO as negative control, while small pilot groups were administered with one of the four CPP-MO compounds by ICV administration at P1 and SC administration at P3 (12 nmoles/dose, see **Table 5**, Materials and Methods). Injected animals were sacrificed at P7 and the brains and the spinal cords were collected for proteomic investigation.

Western blot analysis revealed that all the considered CPP-MO compounds were able to efficiently up-regulate the SMN protein level respect to scr-MO, as illustrated in **Figure 22**. Moreover, all the CPP-MO compounds present a higher activity respect to unconjugated MO-(10-34) (**Figure 22**). In the brain only R6-MO was slightly less efficient than the others, while RXR-MO conjugate was the most efficient in spinal cord, with up to three-fold up-regulation respect to scr-MO (Figure 17 C and D).

Overall we demonstrated that the conjugation of MO with the CPPs enhanced its effect on SMN expression into the CNS after ICV administration.

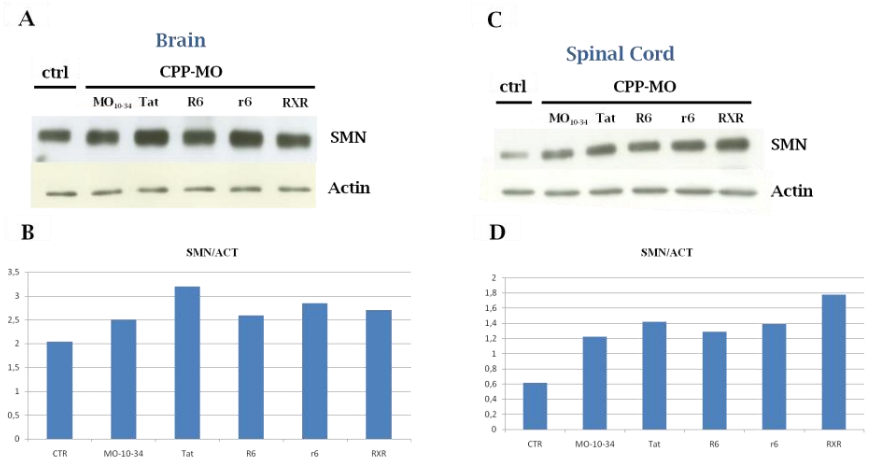

Figure 22: Western blot (**A**) and densitometric analysis (**B**) of brain samples of SMA mice treated with the different CPP-MO conjugates, unconjugated MO or scr-MO by intrathecal administration showed that all the CPP- MO conjugates were more efficacious than unconjugated MO and TAT-MO and RXR-MO were the most efficacious ones. Western blot (**C**) and densitometric analysis (**D**) of spinal cord samples of SMA mice treated with the different CPP-MO compounds, unconjugated MO or scr-MO by intrathecal administration showed that RXR-MO was the most efficacious conjugate as compared to unconjugated MO.

4.7 CPPs-MO EFFICIENTLY INCREASED SMN AFTER SYSTEMIC ADMINISTRATIONIN SMA Δ7 ANIMALS

One of the critical aspect of ASOs approach is their limited intracellular uptake and scarce tissue bioavailability after systemic intravenous injection, in particular for the presence of the blood-brain barrier (BBB), which is a major constraint to their use as therapeutics.

Our aim was to asses if the conjugates CPP-MO were able to cross the BBB reaching the brain and the spinal cord when they were administered systemically, increasing SMN levels.

We used the intravenous injection as systemic administration of MOs.

Per each CPP-MO, one group of treated mice received at P1 12 nmoles of the conjugate (**Table 5**, Materials and Methods section).
CPP-MO injected animals were sacrificed at P7 and their brains and spinal cords were collected for proteomic studies. The results obtained from western blot analysis of the brains demonstrated that MO linked to the TAT or R6 peptide presented a slightly increased capacity to cross the BBB and enter the CNS after systemic intravenous administration. In fact, TAT-MO and R6-MO were able to moderately up-regulate the SMN protein full-length protein level in the CNS of treated animals. On the other hand, the link of MO with the r6 or RXR peptides significantly enhanced the ability of MO to raise the expression of the SMN protein (more than 2.5-fold) after IV injection respect to scr-MO and unconjugated MO (**Figure 23 A** and **B**).

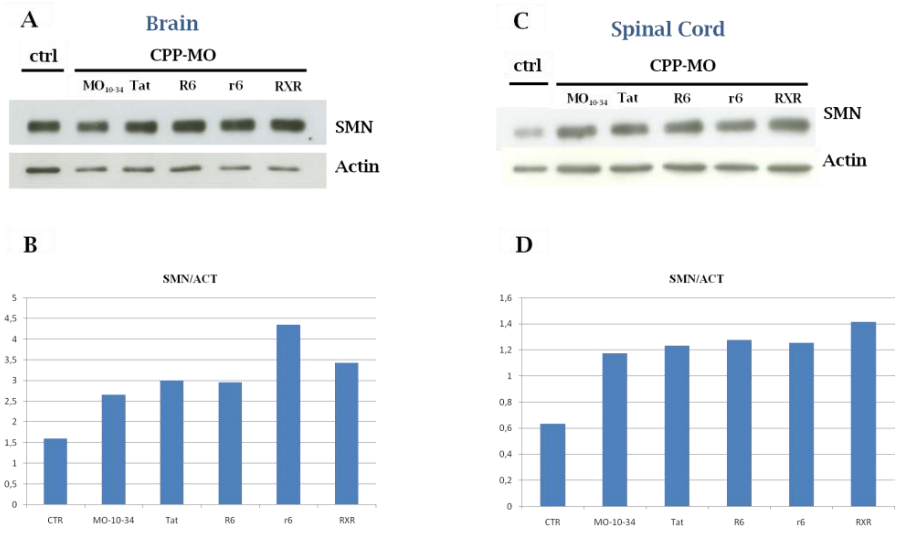

Figure 23: Western blot (A) and densitometric analysis (B) of brain samples of SMA mice treated with the different CPP-MO compounds, unconjugated MO or scr-MO by intravenous injection showed that all the CPP- MO conjugates were more efficacious than unconjugated MO and r6-MO and RXR-MO were the most efficacious ones. Western blot (C) and densitometric analysis (D) of spinal cord samples of SMA mice treated with the different CPP-MO conjugates, unconjugated MO or scr-MO by

intravenous administration showed that RXR-MO was the most efficacious among the tested conjugates.

The results from the spinal cord revealed that the TAT-MO, R6-MO and r6-MO are able to efficiently up-regulate the SMN expression injected animals respect to scr-MO-treated animals and were slightly more efficacious than unconjugated MO. Overall, the RXR-MO compound resulted to be the most potent (**Figure 23 C** and **D**).

These data support our initial idea that CPPs can promote the entrance of MO into the CNS after systemic intravenous injection improving its efficiency. Furthermore, data proved that IV way of delivery is promising and could have comparable or even greater efficacy than CSF direct delivery, with the advantage of a reduced invasiveness.

4.8 NEUROMUSCULAR AND SURVIVAL ANALYSES AFTER CP-MO TREATMENT

The goal of this study is to identify the most efficient CPP-MO that will be further evaluated in a larger cohort of mice to evaluate its effect on survival, neuromuscular function and neuropathology of symptomatic homozygous SMA mice. As a matter of fact, the final aim of the project is to find a strategy to increase the efficacy of MO in symptomatic stages of the disease that usually correspond to the time of diagnosis.

Since all the CPPs tested were able to increase MO efficacy, we selected RXR and r6 for the experiments on homozygous mice, according to their high efficiency and low toxicity. The treatments are ongoing.

We will inject heterozygous SMA mice(P5) with r6-MO or RXR-MO (12nmoles/g) by IP injection. The mice will be sacrificed at p14 and a

western blot analysis to evaluate the SMN level in SC and Brain will be performed in order to evaluate the efficacy of the conjugates.

Subsequently, homozygous SMA mice will follow the same treatments. They will be monitored for survival and motor function, assessed by testing the righting reflex and their performance at the hind-limb test (Tube test) and the rotarod test.

At P6, SMA homozygous animals were clearly unable to perform the righting test. This assay consisted of placing the mouse in supine position on a flat surface and recording the time it took to turn over onto an upright position, or to “right” itself, by standing on all four paws. As expected heterozygous and WT controls were properly able to perform the task.

We performed also the tube test. As expected homozygous SMA animals received a Hind-Limb Score (HLS) of 0 or 1, accordingly to their severe neuromuscular defects.

We investigated also the rotarod assay, and as expected homozygous SMA mice never reach the age to be able to be tested (>1 month of age).

The experiments presented in this section are still under analysis. It is warrants to notice that CPP-MO treated mice that have been up to now examined presented a significantly extended survival. Remarkably they present a reliable amelioration in their neuromuscular abilities as evaluated by behavioral analyses. CPP-treated animals are able to stand and freely ambulate exploring the surrounding environment in a comparable way to healthy wild-type mice. All of them were capable to complete the righting reflex test even after P6 (**Figure 24**).

Figure 24: None of the untreated mice (left) was able to right themselves after postnatal day 6 (P6) while R6-MO treated mice can perform the task even after P6.

CPP-mice received a mean HLS score of 2 or 3 and some of them could even perform the rotarod test in a similar way as wild-type do. Up to now our best results were observed in animals injected with the r6-MO conjugate by local administration. One of these mice has a survival of >4 months, could ambulate and was undistinguishable respect to unaffected wild-type mice. It obtained a HLS score of 3 and could successfully perform the rotarod test (**Figure 25**).

Figure 25: MO treated mice was able to complete the rotarod test and presented an overall aspect similar to healthy wild-type mice.

5. DISCUSSION

Translational medicine advancement has been widely expected for a increasing number of human genetic and neurological diseases. Of these, spinal muscular atrophy (SMA) is the best placed condition to gain on advances in therapeutics development in the near future. Over the past decade several different treatment strategies for SMA, in particular for upregulating SMN level have been developed in several laboratories and now are entering in the clinical stage. Specifically, the use of Antisense Oligonucleotides (ASOs) or small molecules as a tool to correct the alternative *SMN2* splicing seems to be the most interesting strategy.

In particular, the first and only approved drug for SMA, Nusinersen, is an ASO that regulates the splicing of *SMN*2 promoting the production of a functional protein. In this study, we used a modified ASO, called Morpholino (MO), in which the phosphordiamidate link to the Morpholino ring improves its safety and pharmacokinetic profile. The characteristics who make the MO better than ASO are the stability into cells and the resistance to metabolic degradation [163]. The mechanism of action of MO is based on a steric block on the mRNA, that interferes with the assembling of the splicing machinery at the specific mRNA target motif. The efficacy of this process is guaranteed by the perfect complementarity with the target sequence and by the number of nucleotides in the MO sequence. Consequently, a fundamental step in developing a MO-mediated splice-correction approach is the selection of the best target sequence. Furthermore, the delivery of a combination of two different MO oligomers was not been yet tested *in vitro* or *in vivo*.

The primary goal of this project was to find and test new MO sequences with a better efficiency in comparison to MO-(10-34). We

designed four novel oligomers (called MO-A, B, C, D) to match the ISS-N1 region, in the SMN2 intron 7. However, while MO-A and MO-B oligomers are complementary to the target motif, MO-C and MO-D sequences bind it only for the first 10 nucleotides.

Usually, the oligonucleotides should prevent the assembly of the splicing machinery, leading to exon 7 retention, while, for MO C and MO D, we suppose that their free oligonucleotide tail can cause a more efficacious steric block effect, preventing exon 7 splicing. The new sequences were tested *in vitro* on wild-type human cell lines and on iPSCs reprogrammed from fibroblasts of SMA type 1 patients. We tested the novel sequences also on iPSC-derived MNs. We observed that there was a relative variability in the ability of the different MO sequences to up-regulate the SMN protein levels. Of these, MO-B and MO-D were the most potent compounds, allowing the greater expression of gems in cell nuclei and full-length SMN. Due to the positive results of these two sequences, we decided to combine them in a single administration, maintaining the same total dosage. Data obtained showed that the co-co-administration of MO-B plus MO-D was more effective than the treatment with a single MO oligomer, and also than the already validated MO-(10-34). The collection of iPSCs at different time points after treatment with MO-B, MO-D and MO B+ MO D was useful to analyze the SMN protein levels during time. All the treatments showed an increase of SMN also after 21 days after the nucleofection, even if the most efficient time point was 72 hours.

Remarkably, we demonstrated also that MO B+D combination is able to increase SMN protein and rescue neuropathological features of SMA iPSC derived MNs, the cells that are selectively affected in SMA.

The sequences that performed better *in vitro* were tested *in viv*o in heterozygous SMAΔ7animalsin respect to that treated with the MO-(10-

34) oligomer. Animals were injected intracerebroventricularly (ICV)and subcutaneously (SC) at P1 and again SC at P3. The findings generated in *in vivo* settings were in line to those demonstrated *in vitro* and they confirmed that the co-administration of MO B + MO D was more effective than the treatment with MO B or MO D alone in up-regulating the SMN full length protein in the brain and spinal cord of SMA mice. Furthermore, after the administration of MO B + MO D, the SMN levels were higher in the spinal cord of treated animals respect to those treated with MO-10-34.

We hypothesized that the reason of the enhanced efficacy of MO B and MO D could be the different way in which they match the ISS-N1 region. In fact, while MO B perfectly matches the target sequence, the MO D has an unpaired nucleotide tail that could account for an increased steric block action of MO-D itself. Our idea is that this strategy has two positive effects on the clinical advancement of this therapy. From one side we have an increase of the efficacy of the MOs, while on the other side these sequences can achieve the same biological effects using a lower dose. Our promising results should be validated by conducting other *in vivo* experiments in order to test the toxicity and the efficacy on the SMA phenotype of these new MO sequences.

Another goal of our study is to improve the poor *in vivo* bioavailability of ASOs. In fact, despite the positive aspects of ASOs, such as their suitability to treat a wide range of human diseases, they have some limitations that have been improved by chemical modifications. The poor stability and their toxicity have been enhanced substituting the phosphorothioateribose backbone with a phosphorodiamidate-linkage morpholino backbone, making them refractory to metabolic degradation. However, the route of administration and the distribution in

human tissues are still the major obstacles of this therapeutic approach. The critical step of ASOs in reaching their target is the crossing of different biological barriers, like endothelial cells of small blood vessels, the plasma membrane and the nuclear membrane. But the most difficult passage is through the brain blood barrier(BBB) to reach the Central Nervous System (CNS). The structure of the BBB makes it highly selective for the transfer of macromolecules from the blood stream to the CNS. This is the reason why large and hydrophilic macromolecules are usually injected directly into the cerebrospinal fluid to act on the CNS.

A promising strategy to facilitate the delivery of ASOs is the application of cell-penetrating peptides (CPPs). We explored if the conjugation of CPPs to MO oligomers allow them to easily cross the BBB after a systemic non-invasive intravenous injection and promote their efficacy and distribution into the CNS of SMA mice.

To validate our thesis, we first conjugated MO-10-34 to the TAT peptide, as it is the best studied peptide to date and allows the penetration of different molecules through a mechanism of endocytosis. Moreover, the positive charge of pTAT can help the biodistribution through the brain [160].

Another critical issue is the therapeutic window of the disease. All research groups agree on the fact that the early administration of MO is more effective than late administration. But the presymptomatic diagnosis of the pathology is not always possible. For these reasons we decided to treat the mice at P5 and P7. Early results of the administration of unconjugated MO at these ages showed a reduction in amelioration of the phenotype and a reduced extension in the survival compared to the early delivery (P0 and P3). The explanation of

these data could be due to the leakiness of the BBB of the neonatal mice.

The low biodistribution of unconjugated MO and the low efficacy of the P5/P7 treatment allowed us to test whether the conjugated TAT-MO is more efficient that unconjugated MO when they were administered at P5 and P7.

We treated two groups of homozygous affected SMA mice by ICV injection plus SC injection at P5 and P7 and with another SC injection at P7 and we compared these results with the two obtained using the unconjugated MO. The first group received a dosage of 6 nmoles per injection. Simultaneously, the other group was treated with 12 nmoles per injection of TAT-MO conjugate, to verify if the response could be dose-related. Data showed a modest increase in lifespan in mice treated with a lower dose of TAT-MO compared to unconjugated MO, while a significant increase in survival for the group treated with 12 nmoles of the conjugate was observed.

Interestingly, we noted that the purity of the TAT peptide strongly influenced the outcome of treated mice. Indeed, we observed a high toxicity (almost all the mice died within few days after the treatment) with a batch of TAT peptide that next revealed a lower purity degree, while no lethal toxicity was found in the mice groups treated with the other batches. We excluded the data obtained with the batch that was not well purified. As described by Moulton[79, 154, 164],if TAT was administered as a free peptide, it showed a dose-dependent effect that should not exceed the dose threshold to ensure safety. Thus a chromatography purification is necessary to eliminate the excess of free peptide.

Our positive results allowed us to choose TAT and three other cationic CPPs (named R6, r6 and RXR) to be conjugated with MO-10-34 and to

test their ability to cross the BBB, increasing the production of full-length SMN protein.

The R6 and r6 polyarginine CPPs are shorter and simpler than TAT [93]. They are made up by six covalent bounded arginine residues and differ only for the configuration of the amino acids: L enantiomeric configuration for R6 and D enantiomeric configuration for r6. Their efficiency is expected to be comparable to that of TAT peptide [114].

(RXRRBR)2 XB is the other peptide chosen because it has been reported to be able to cross the BBB and to successfully deliver ASOs to the brain and cerebellum of mice after a systemic administration [165]. The positive aspects of this peptide are its good safety and efficacy profile also *in vivo*, with no signs of toxicity reported. All these data provided a good background for the selection of the most efficient CPP for the delivery of our MO sequence.

At first we treated a small pilot group of heterozygous mice with the four conjugates, the unconjugated MO and scr-MO (as control) in order to select the most efficient one. We tested two routes of administration: the local and the systemic one and we quantified the SMN protein levels in brain and spinal cord by western blot. After ICV injection, all the four CPP-MO conjugates increased the SMN levels more than unconjugated MO. In particular, RXR-MO was the most efficacious in the spinal cord (up to 3-fold compared to scr-MO) and its efficacy in the brain was comparable to that of the other conjugates. After IV injection, the best results were obtained after r6 and RXR conjugates administration in the brain (more than 2.5-fold), while in the spinal cord the most efficacious conjugate was RXR-MO. Our results showed that this strategy can be suitable to enhance the pharmacokinetic profile of MO, increasing its cell and tissue penetration after a local injection and

to cross the BBB more efficiently than unconjugated MO, growing its delivery to the CNS, after a systemic administration.

Probably, the ability of the conjugates to increase their biodistribution and the cellular uptake is due to a longer retention of MO into cells, allowing a longer timespan between one intrathecal injection and another. This would result in less complication and side effects related to the procedure and considerably less discomfort to the patients.

Moreover, the different capacity of the CPPs to cross the biological barriers is a fundamental characteristic in the selection of the most efficient one for the development of a non-invasive delivery strategy of MO oligomers. Further experiments on symptomatic homozygous SMA mice will be performed in order to obtain stronger evidence of the efficacy of our approach.

Another issue is the route of administration of these conjugates. In fact, Hua et al. emphasized the importance of peripheral administration, while Burghes group supported the local CNS administration as the most efficacious method to restore the phenotype of treated mice [74, 80].

Previous data from our lab showed that a single ICV injection of unconjugated MO was able to modify SMN2 splicing, increasing the survival of affected mice [83]. But the combination of local (ICV) and systemic (SC) injections determined an amelioration of NMJs, increased the MNs number and improved the muscular trophism.

For all these reasons, mice will be treated with a local ICV injection and/or with a systemic IP injection, and we will evaluate the effect of the conjugates on survival and motor functions. The choice of symptomatic SMA mice is done in order to mimic the real therapeutic window in humans. In fact, SMA in rarely diagnosed in the pre-symptomatic phase of the pathology. This means that we cannot

replicate in humans the positive results obtained in pre-symptomatic SMA mice. These experiments are still ongoing but our preliminary results showed an improvement of lifespan and motor performance of treated SMA mice, compared to the untreated ones. In particular, treated mice showed increased lifespan, could walk and explore the environment. They could perform different functional tests, like the righting reflex test after P6, achieved a Hind-limb Score (HLS) score from 2 to 3 and could complete the rotarod test.

The best result we obtained to date in symptomatic mice is the one with r6-MO conjugate, administered by ICV delivery. One of these mice lived >4 months and showed a striking improvement in motor performance. It received a HLS score of 3, could successfully perform the rotarod test and behaved like unaffected littermates.

Recently, it has been reported that the new family of Pip6 (a-f) CPPs could mediate the delivery of MO oligomers. In particular, Hammond and colleagues demonstrated that Pip6a could mediate the delivery of MOs in the CNS of SMA mice, increasing their lifespan [166].

When the symptomatic mice were treated with Pip6-MO by IV injection at P0, their lifespan increased of more than 450 days (average survival = 167 days), which is one of the best results reported in the literature to date. The interesting discovery was the dose-related response: when the pups were treated with a low dose of conjugate (10µg/g), which correspond to 1nmol/g, their motor functions significantly improved. This dose is five times lower than the doses used in other studies and ten times lower than the dose used in our work, suggesting that this peptide is particularly efficient in crossing the BBB compared to the other tested CPPs. Since the critical part of the SMA therapy is to reach the CNS of patients at all age, Hammond and colleagues tested this compound in unaffected adult SMA mice harboring SMN2 allele. They

administered twice 18 µg/g of Pip6a-MO by tail vein injection in 7.5 wk old mice, and collected the tissues 7 days after the treatment. The analysis of FLSMN2 transcripts revealed an increase in brain, spinal cord, skeletal muscle and liver. However, the SMN protein levels were augmented in skeletal muscle and liver only, maybe due to the sensitivity of SMN protein detection method. The difference between our study and Hammond's study is that we are testing all the compounds on affected SMA mice at P5 using a single administration dose and we will evaluate not only the increase of SMN levels, but also we will monitor the survival rate, the behavior and neuropathological analysis, in order to prove also the survival of MNs in spinal cord.

Since SMA infants are usually diagnosed after symptom onset, the treatment of the disease in a symptomatic phase is a critical step for the translation of the therapy in a clinical setting. Our preliminary results opened the possibility to widen the therapeutic window with our new approach.

6. CONCLUSIONS

Globally, the results of our experiments strongly support the feasibility of the MO-mediated therapy for SMA. The efficacy of MO has been improved with two techniques: the optimization of the target sequence and the co-administration of two different sequences. The results of these approaches produced a high percentage of full-length SMN protein. Moreover, the cellular uptake and the pharmacokinetic profile of MO have been enhanced by the conjugation of our validate sequence with CPPs, opening the possibility of a non-invasive administration of MOs.

Our future plans are:

1) to complete the experiments on treated symptomatic mice at P5 in order to select the best CPP, evaluating their phenotype and the survival rate. Behavior analysis will be performed as previously reported [53, 74, 80].
All injected animals will be monitored daily for clinical signs of disease such as morbidity, mortality and weight by blind observers. The average life span of SMA mice is 13.6±0.7[167], Kaplan-Meier survival analysis and log-rank test will be used for survival comparisons.

2) to perform neuropathological analysis of symptomatic treated mice with methodologies previously described [53, 83] to define if the treatment ameliorates the cellular phenotypes, reducing the decline in motor neuron cell counts in the spinal cord and improving muscle trophism. In particular, the count of the number and the size of motor neurons will be performed analyzing the ventral horn of the spinal cord and the axons in the ventral roots.

SMA mice show a loss of muscle mass, and their myofibers are typically small with no change in the number of nuclei per fiber and no increase in the number of centrally localized nuclei [168]. Furthermore, the SMA mice present a reduction of myofibers possibly linked to the loss of function of SMN protein in muscle causing a decline of muscle regenerative capacity. Proximal and distal nerves and overall muscle pathology will be assessed, including total cross-sectional area and the diameter and number of myofibers. Myofibers from each muscle will be randomly selected the cross-sectional area of each myofiber will be measured and the average myofiber size per muscle for each animal will be calculated. In addition, we will perform morphometric analyses of the neuromuscular end plate by assessing labeled neuromuscular junctions (NMJs) on the hind limb and intercostal muscles of CPP-MO treated SMA mice. The NMJ from each muscle will be randomly selected and assessed under the microscope to quantify the number of collapsed NMJ for each muscle group per animal.

These two points will allow us to understand which conjugate could be translated in clinic even if some tests in larger animals should be performed to confirm the data obtained in mice.

3) to test new peptides recently described in the literature, exploring the the Neurotoxin of Clostridium TetaniC fragment (TTC) and on a new family of synthetic cationic CPPs identified as Pip.
The neurotoxin, produced by the bacterium Clostridium Tetani, is produced as a single polypeptide and then cleaved into a light (L) chain and a heavy (H) chain which are linked to each other by a disulfide bond.

The toxin specifically binds to neurons, especially to MNs [388]. It enters in neurons at the level of NMJs and once it reaches the dendrites, it can spread to adjacent neurons through synapsis. The essential part of this toxin is the C-terminal of the H chain (also called Tetanus Toxin C fragment or TTC), which is able to bind neurons without any toxic effect. Due to this ability, TTC can be conjugated to different molecules [169-171] and can be used as a carrier to target specific neurons.

We believe that TTC-mediated MO oligomers delivery to MNs could give positive results, and in general this strategy can be useful to deliver therapeutic molecules to CNS by bypassing the BBB for the treatment of neurological diseases.

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